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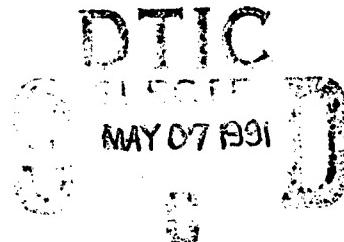
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**1989 TOXIC HAZARDS  
RESEARCH UNIT  
ANNUAL REPORT**

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## **TECHNICAL REVIEW AND APPROVAL**

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE COMMANDER**

JAMES N. McDUGAL, Maj, USAF, BSC  
Deputy Director, Toxic Hazards Division  
Harry G. Armstrong Aerospace Medical Research Laboratory

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<p>This report has been prepared as a review of the October 1988 through September 1989 activities of the Toxic Hazards Research Unit. Research activities largely focused on the toxicity of chlorotrifluoroethylene oligomers and the acid metabolic products of this hydraulic fluid material. Initial acute studies also were conducted on a silahydrocarbon hydraulic fluid. Physiologically based pharmacokinetic models were developed, refined, or more substantially validated for several chemicals of Air Force and Navy interest. These included hydraulic fluids, organophosphates, and combustion products. Both <i>in vivo</i> and <i>in vitro</i> studies on chloropentafluorobenzene, a chemical defense training material, were conducted or subcontracted, and efforts were begun to develop the equipment needed for additional studies using primates. Neurotoxicity assessments were conducted for jet engine oils and a model was developed to assess the dermal penetration of a neurotoxic compound found in the combustion products of another lubricating oil. In addition, studies were conducted to characterize the distribution of test agents in inhalation exposure chambers and to describe the effects of animals on such distributions. The review of work described here includes partially completed efforts that will be reported further in subsequent annual reports.</p>			
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**Chlorotrifluoroethylene Oligomer**  
**Cyclotriphosphazine**  
**Dichlorotetrafluorobenzene**  
**Fluoride**  
**Genotoxicity**  
**Inhalation**  
**Inhalation Chamber**  
**Initiation/Promotion**  
**O,O'-Diethylmethylphosphonite**  
**Organophosphate**  
**Paraoxon**  
**Perhalocarboxylic Acids**  
**Physiologically Based Pharmacokinetic Model**  
**Polychlorotrifluoroethylene**  
**Silahydrocarbon**  
**Triethylborane**  
**Trimethylolpropane Phosphate**  
**3.1 Oil**

## PREFACE

The 26th Annual Report of the Toxic Hazards Research Unit (THRU) presents research and support efforts conducted by NSI Environmental Sciences (NSI-ES) on behalf of the U.S. Air Force and the U.S. Navy under Contract Number F33615-85-C-0532. This document represents the fourth report for the current THRU contract and describes accomplishments from October 1988 through September 1989.

Operation of the THRU under this contract was initiated in January 1986 under project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations," Task 00, Toxic Hazards Research, Work Unit Number 63020001. This research effort is co-sponsored by the Naval Medical Research Institute, Toxicology Detachment, Navy Work Unit Number M0096-004-0006, "Criteria for Exposure Limits in the Navy Operational Environments." The objectives of the research program are to identify and characterize the toxic effects of chemicals and materials that are of operational interest and concern to the Air Force and Navy. This coordinated dual-service program generates scientific information from which computer simulation models of toxicity can frequently be developed to drive the experimental design and to assist in risk assessments.

The Toxic Hazards Division, Harry G. Armstrong Aerospace Medical Research Laboratory technically monitored this contract. Melvin E. Andersen, Ph.D., Acting Director of the Toxic Hazards Division/Senior Staff Scientist, served as the Contract Technical Monitor until 9 January 1989. Lt Col Harvey J. Clewell, III, Deputy Director of the Toxic Hazards Division, served as the Contract Technical Monitor from 9 January until 13 July 1989. Lt Col Michael B. Ballinger, Director of the Toxic Hazards Division, has served as the Contract Technical Monitor since 13 July 1989.

CDR David A. Macys, MSC, USN, was the Director of the U.S. Navy portion of this contract during the period of this report.

Raymond S. Kutzman, Ph.D., was the NSI-ES Program Manager for the THRU contract during the period of this report. The preparation of this report represents the combined efforts of the NSI-ES staff of the THRU.



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**SECTION 1**  
**LIST OF ABBREVIATIONS**

AChE	-	Acetylcholinesterase
AFWAL/MLBT	-	Air Force Materials Laboratory
ALT	-	Alanine aminotransferase
AST	-	Aspartate aminotransferase
ATPase	-	Adenosine triphosphatase
BCPES	-	Bicyclophosphorus esters
BW	-	Body weight
CHLF	-	Chloroform
CHO	-	Chinese hamster ovary
CoA	-	Coenzyme A
CPFB	-	Chloropentafluorobenzene
CTFE	-	Chlorotrifluoroethylene
CTFE;3.1 oil	-	Chlorotrifluoroethylene oligomer
CTP	-	Cyclotriphosphazene
CV	-	Coefficients of variation
DCFB	-	Dichlorotetrafluorobenzene
DEN	-	Diethylnitrosamine
EDTA	-	Ethylenediaminetetraacetic acid
F-344	-	Fischer 344
GABA	-	Gamma-aminobutyric acid
GC	-	Gas chromatograph(y)
GGT	-	Gamma-glutamyltransferase
G6Phase	-	Glucose-6-phosphatase
GST-P	-	Glutathione-S-transferase
H&E	-	Hematoxylin and eosin

HGPRT	- Hypoxanthine-guanine phosphoribosyl transferase
HP	- Hewlett-Packard
IP	- Intraperitoneal
IRP	- Installation Restoration Program
KB	- Diisopropylaminoethanol
LT	- Bis (diisopropylaminoethyl) phosphonite
NCI	- National Cancer Institute
NTE	- Neurotoxic esterase
NZW	- New Zealand white
ODC	- Ornithine decarboxylase
OP	- Organophosphorus
OPIDN	- Organophosphate-induced delayed neuropathy
OW	- Organ weight
PAS	- Periodic acid/schiff
PB	- Phenobarbital
PB-PK	- Physiologically based pharmacokinetic
PFDA	- Perfluoro-n-decanoic acid
Pv	- Vapor pressure
QL	- O-Ethyl-O'-(diisopropylaminoethyl) methylphosphonite
RH	- Relative humidity
SCE	- Sister chromatid exchange
SEM	- Standard error of mean
SHC	- Silahydrocarbon
TCD	- Thermal conductivity detector
TCP	- Tricresylphosphate
TEM	- Transmission electron microscopy
TEP	- Triethylphosphonite
THRU	- Toxic Hazards Research Unit

TISUB	- Total ionic strength urinary buffer
TMP-P	- Trimethylolpropane phosphate
TOCP	- Triorthocresylphosphate
TR	- <i>O,O'</i> -Diethylmethylphosphonite
TRO	- <i>O,O'</i> -Diethylmethylphosphonate
USAF	- United States Air Force
YL	- <i>O</i> -Ethylmethylphosphinate
ZS	- Ethanol

## SECTION 2

### INTRODUCTION

The research activities and support efforts of the Toxic Hazards Research Unit (THRU) are conducted as a continuous program independent of contract year. Research areas established by the U.S. Air Force and U.S. Navy are supported by individual studies. The papers in this report generally summarize the developments in a selected study area. The papers are organized to address work on Air Force fuels and lubricants, which include a number of studies on chlorotrifluoroethylene oligomers (CTFE), components of a candidate, nonflammable, hydraulic fluid; physiologically based pharmacokinetic (PB-PK) modeling of several chemicals and studies that support the modeling efforts; studies on materials associated with chemical defense, both to study the kinetics of organophosphate agents and to develop nontoxic training agents; studies requested by the U.S. Navy that included toxicology assessment of a hydraulic fluid, lubricating oils, combustion products, and the advanced characterization of inhalation chamber toxicant distribution; and work conducted at the request of the U.S. Army, through the U.S. Air Force, on the toxicity of a potential breakdown product of an organophosphorus chemical munition component. Many of the papers in this report reflect progress in more than one of the indicated areas of research. Effort has been made to group them such that information in particular subject areas can be readily extracted.

Research on Air Force fuels and hydraulic fluids was dominated by studies of CTFE and the corresponding carboxylic acids that are the suspected toxic metabolites. A mixture of trimers and tetramers of CTFE has been proposed as a hydraulic fluid for advanced aircraft systems. These varied carbon chain lengths and arrangements of the halogens permit alteration in the physical properties of the material to meet engineering requirements. These properties also present challenges when generating exposure atmospheres for inhalation toxicology studies. Generation methods were developed that would provide treatment atmospheres that remained chemically comparable to the parent material. In addition, analytical procedures were developed to determine the mass concentration and the distribution of oligomers in both the vapor and aerosol phases of the exposure atmosphere, and to determine the oligomer distribution as a function of aerosol particle size.

Data from the studies on CTFE ultimately will be used for a risk assessment on this fluid. To aid in the development of the most appropriate data and to permit quantitative extrapolation from test animals to humans, a PB-PK model is under development for CTFE. To validate the model, methodology was developed for the analysis of the CTFE in animal tissues, blood, and urine. The prevailing hypothesis of CTFE toxicity is that the acid metabolites of CTFE, formed at various rates in different animal species, result in hepatic peroxisome proliferation. Therefore, methods were

developed for the analysis of both the parent compound and the metabolites. Because removal of a fluoride molecule from the parent compound is required for acid formation, the excretion of fluoride from treated animals also was assessed. Data to support the development of the PB-PK model were accumulated from several studies of CTFE and its metabolites, including a second subchronic inhalation study conducted at 10, 50, and 250 mg CTFE/m<sup>3</sup> to establish a no-effect concentration.

Studies were initiated to determine the persistence of the CTFE-induced lesions in the rodent following termination of exposures and to assess the potential of CTFE to induce hepatic lesions in diverse species by dosing hens. Investigations of the suspected toxic acid metabolites of CTFE included acute lethal dose studies. The results of these studies were used, along with the preliminary PB-PK model simulations, to establish doses for long-term repeated oral dosing studies and for an initiation/promotion study of CTFE trimer acid. When completed, the data from these studies are expected to provide a sufficiently comprehensive understanding of the toxicity of CTFE such that a cogent risk assessment can be developed for Air Force applications.

Initial acute studies were completed on silahydrocarbon, which is a nonflammable hydraulic fluid with minimal thermal expansion properties. These studies included acute oral, dermal, and inhalation treatments as well as assessment for skin sensitization and skin and eye irritation testing.

A number of the physiologically based pharmacodynamic modeling papers in this report stem from the quantitative assessment of hepatocytic damage and proliferation. These are important parameters to be included in a PB-PK model for hepatocarcinogenesis. A study was conducted to determine the clearance of alanine aminotransferase from the blood of untreated animals as a first step in determining the effectiveness of using the activity of this serum enzyme as an index of liver cell necrosis. Once established, this index could be used to estimate liver damage during repeated treatment scenarios without sacrificing the study animals. In anticipation of assessing the tumor initiation and/or promotion potential of selected chemicals and the incorporation of the cell proliferation kinetics into the appropriate pharmacodynamic models, a study was conducted using a known initiator and a known promoter, diethylnitrosamine and phenobarbital, respectively. Following treatment, foci of altered liver cells were detected with gamma-glutamyltranspeptidase.

To assist in the health risk assessment for exposure of personnel to organophosphate chemicals, data were developed for incorporation into a PB-PK model for paraoxon. Laboratory work included the *in vitro* determination of the solubility of paraoxon in selected biological tissues. These data then were compared to values calculated from information in the literature. To ensure proper training of personnel for barrier protection from hazardous chemicals, a relatively nontoxic material is being examined as a training agent. Chloropentafluorobenzene (CPFB) has been identified as a potential candidate chemical. In training scenarios, garbed and masked personnel will

be exposed to CPFB. Upon completion of the training exercise their breath will be analyzed and their exposure history calculated using a PB-PK model. To be used for this purpose, a chemical must be shown to be safe, should excessive exposure occur, and a highly accurate PR-PK model must be developed. Two CPFB repeated-inhalation exposure studies were conducted using rodents, one for 21 days and the other for 90 days. Data from these studies were used to establish the relative toxicity of CPFB and to refine the rodent model for this compound. Because humans will be exposed to this material, studies are planned that will permit validation of the PB-PK model in primates. To this end, a custom latex mask with minimal dead space is being developed to expose monkeys to CPFB and to measure accurately the exhalation of the chemical following the exposure.

The acute delayed neurotoxicity potential of two jet engine oil formulations that contain either tricresyl phosphate or its *ortho* derivative was examined in leghorn hens. Although the tricresyl phosphate content of these oils was found to be low (~3%), single exposures to a neurotoxic organophosphorus compound can result in axonal damage after a delay of 8 to 10 days. Trimethylolpropane phosphate (TMP-P) is another compound of interest because of its neurotoxic potential. This compound is formed as a result of the pyrolysis of trimethylpropane triheptanoate-based lubricants used extensively in commercial and military jet propulsion systems. TMP-P belongs to a class of compounds referred to as bicyclophosphorous esters, which tend to bind to selected central nervous system receptors. Interestingly, a sex difference in sensitivity has been reported following dermal treatment of rodents with this compound. Therefore, studies were conducted to assess this gender difference and to develop data for a PB-PK model for this compound.

To minimize nonbiological reasons for variation in the doses animals receive within study groups, the distribution of the test agent within an inhalation chamber must be uniform. However, such conditions do not always prevail. Therefore, it is necessary to characterize the distribution of test material within the chamber. These characterizations are used to determine if the inhomogeneities are sufficient to impact the study and to provide a basis from which to judge the effectiveness of modifications to the chamber. Several approaches to characterize the distribution of test material in the 0.7-m<sup>3</sup> chambers used by the THRU are described. A mathematical model of mixing characteristics in dynamic flow reaction vessels was used to estimate the effective mixing volume of these chambers. In addition to the geometric configuration of the inhalation chamber affecting its flow and mixing characteristics, there are also thermal effects to be considered. These thermal effects are introduced when the temperature of the chamber wall is different than that of the exposure atmosphere and the heat source provided by each test animal. The effect of wall temperature on the flow structure within these chambers was investigated extensively.

Because of the inhalation capabilities available at the THRU, the U.S. Army requested that selected studies on several breakdown products of O-ethyl-O'-(diisopropylaminoethyl)

methylphosphonite, one of the components of their chemical munitions, be conducted utilizing these facilities. The study reported herein is on the virtual acute inhalation toxicity of O,O'-diethylmethylphosphonite, which very rapidly hydrolyzes to O-ethylmethylphosphinate and ethanol in atmospheres of normal relative humidity.

In addition to the conduct of scientific investigations, the THRU conducted two toxicology conferences. The 19th Conference on Toxicology: Contemporary Initiatives in Quantitative Toxicology was held 1 through 3 November 1988. A Conference on Occupational Health Aspects of Advanced Composite Technology in the Aerospace Industry was held 6 through 9 February 1989. The proceedings of these conferences are published under separate Technical Report covers and will not be discussed in this report.

Other contract activities not specific to research efforts are covered in the appendices of this report. Appendix notation is not designed to diminish the importance of these efforts; they describe, in fact, the administrative and support requirements that are critical for the conduct of the studies appearing in the body of this report.

## SECTION 3

### TOXICOLOGY EVALUATIONS OF AEROSPACE HYDRAULIC FLUIDS AND FUELS

#### 3.1 PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL OF INHALATION EXPOSURE OF FISCHER 344 RATS TO CHLOROTRIFLUOROETHYLENE OLIGOMERS

A. Vinegar, C.S. Seckel, D.L. Pollard, H.C. Higman, E.R. Kinkead, M.E. Andersen<sup>a</sup>, and R.B. Connolly

#### ABSTRACT

A mixture of chlorotrifluoroethylene (CTFE) oligomers is being considered as a candidate hydraulic fluid. A preliminary physiologically based pharmacokinetic (PB-PK) model for CTFE was developed that allows prediction of the distribution of CTFE in body tissues during different exposure scenarios and, once fully validated, prediction of the pharmacokinetic behavior in humans. The model will be used for risk assessment and thereby will play an important role in cost-benefit decisions concerning the use of CTFE. Pharmacokinetic data were collected after exposure of male Fischer 344 (F-344) rats to 0.25 mg CTFE/L for 6 h or to 0.25 or 0.01 mg CTFE/L for 6 h/day, 5 days/week for 85 days. Gas chromatograms of CTFE showed two groups of peaks. These lighter and heavier molecular weight components of CTFE were followed pharmacokinetically as separate compounds, designated as Groups I (trimer) and II (tetramer) oligomers, respectively, using identical model structure but with appropriate parameter values for each group. Partition coefficients were estimated from tissue:blood concentration ratios at the end of 90 days of exposure. A first-order metabolic rate constant was estimated based on the rate of fluoride appearance in the urine and an assumption of 1.5 mol of fluoride released per mole of CTFE metabolized. An unusual aspect of model structure was the explicit description of two fat compartments with different perfusion characteristics. Relative concentrations of Groups I and II oligomers in venous blood, liver, and fat after a single 6-h exposure remained in proportion to the original inhaled vapor concentrations. However, after multiple exposures, tissue concentrations of Group II oligomers were relatively and absolutely higher than those of Group I. The combination of high fat:blood partition coefficient and poor perfusion/diffusion characteristics resulted in a long postexposure retention of CTFE in fat, which drove continuous blood and tissue exposure during the postexposure period. This study provides insights of the pharmacokinetic behavior of CTFE and illustrates the development of a model that simulates the pharmacokinetic behavior of CTFE after acute or subchronic inhalation exposure.

<sup>a</sup> AAMRL/TH, Wright-Patterson Air Force Base, OH

The pharmacokinetic data are consistent with the hypothesis that the toxicity of CTFE is related to the preferential accumulation of longer chain oligomers.

#### **INTRODUCTION**

CTFE (3.1 oil) is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectric strength. These properties make CTFE promising for potential use in hydraulic fluid systems.

The liver was shown to be the principal target organ in subchronic inhalation studies of rats to CTFE. The kidney also was affected, though to a lesser extent. Males were more sensitive than females. A no-effect level for males of 0.01 mg/L for an exposure regimen of 6 h/day, 5 days/week for 13 weeks was identified based on light and electron microscopic, hematologic, and in-life data (Section 3.3, Subchronic Inhalation Toxicity Studies on 3.1 Oil at Concentrations of 250, 50, and 10 mg/m<sup>3</sup>).

An interesting and potentially important observation from the 13-week rat inhalation studies was the accumulation of hepatic peroxisomes. Both peroxisome number and the associated activity of peroxisomal beta oxidation of palmitoyl coenzyme A showed concentration-dependent increases (Section 3.3, Subchronic Inhalation Toxicity Studies on 3.1 Oil at Concentrations of 250, 50, and 10 mg/m<sup>3</sup>). Many compounds that cause peroxisome proliferation in the rat are also hepatocarcinogenic (Reddy and Lalwani, 1983). Although there are no data directly indicating that CTFE is a rodent carcinogen, these data on peroxisome proliferation are suggestive of this possibility. Also, to date, there is no direct evidence that chemicals that cause peroxisome proliferation and that are hepatocarcinogenic in rats are also hepatocarcinogenic in humans.

Blood and tissues were taken during the subchronic studies to obtain pharmacokinetic data that were used to develop a PB-PK model. The PB-PK model for CTFE allows prediction of the distribution of CTFE in body tissues at different exposure concentrations and, once fully validated, prediction of the pharmacokinetic behavior in humans. The model then will be used for risk assessment and thereby will play an important role in cost-benefit discussions on the use of CTFE. The pharmacokinetic behavior of CTFE in the rat and the development of the PB-PK model that simulates its behavior are presented herein.

#### **METHODS**

##### **Test Substance**

The test substance was halocarbon 3.1 oil (CTFE), a mixture of oligomers of chlorotrifluoroethylene. Capillary gas chromatography (GC) with electron capture detection showed two distinct groups of peaks, hereafter called Groups I (trimer) and II (tetramer). GC/mass

spectrometry showed that Group I consisted of C5 through C7 oligomers, with most of the mass being C6. Group II was primarily C8 oligomers, with minor C7 and C9 components.

#### ***Exposure Regimen***

Male F-344 rats were exposed 6 h/day, 5 days/week for 13 weeks to concentrations of 0.25 and 0.01 mg CTFE/L, free of additives and having a 55:45 ratio of Group I and II oligomers. Additional male rats were exposed only once for 6 h at 0.25 mg/L. A more complete description of the exposure regimen is provided in Materials and Methods, Section 3.3.

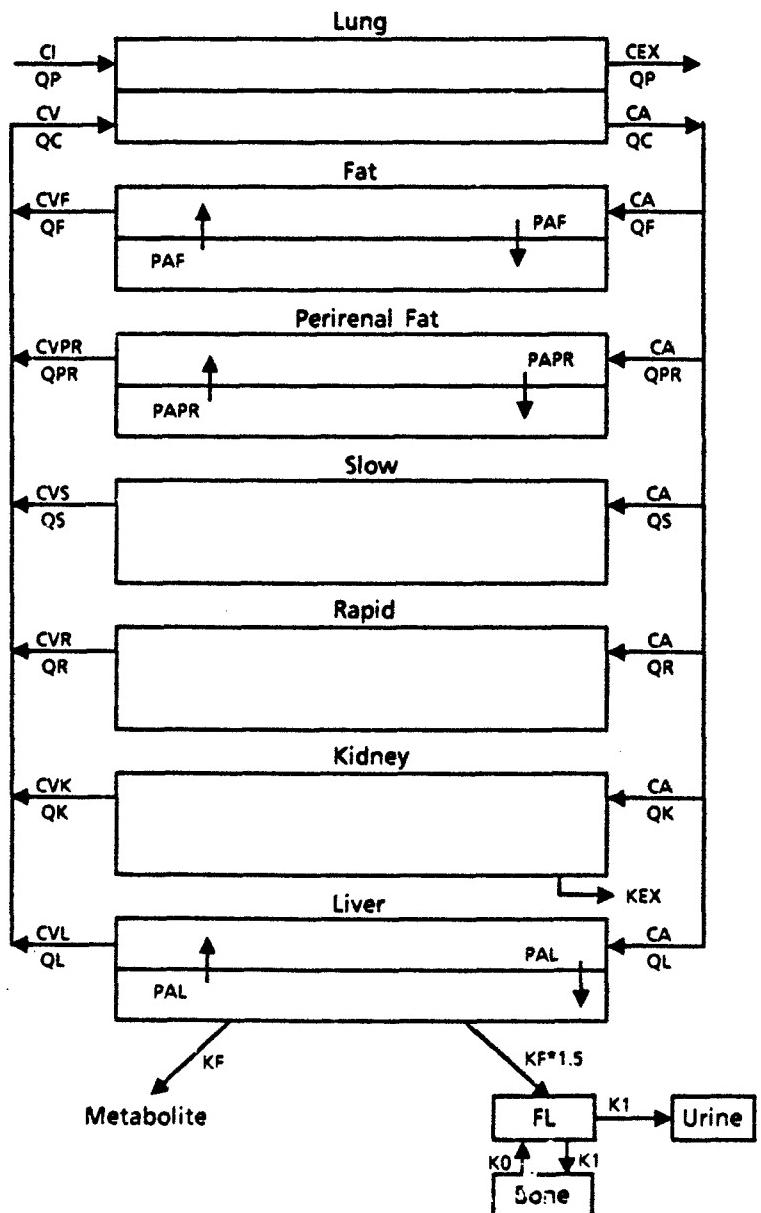
#### ***Analysis of Biologic Samples***

Methods of analysis essentially were the same as reported in the 1988 THRU annual report (Pollard and Higman, 1989).

#### ***MODEL DEVELOPMENT***

A PB-PK model was written in SimuSolv (Mitchell & Gauthier Associates, Concord, MA), a FORTRAN-based continuous simulation language with optimization capabilities, and run on a VAX 8530 (Digital Equipment Corp., Maynard, MA). The general form of the model (Figure 3.1-1) follows that of Ramsey and Andersen (1984). Physically separate, although structurally identical, models were developed for oligomer Groups I and II. Fat, lung, liver, kidney, and rapidly perfused and slowly perfused organ groups each were described explicitly by mass balance equations. Also included were the diffusion limitation between blood and liver and blood and fat and the description of two fat compartments. These two compartments were simulated as occupying 0.4 and 0.6 of total fat volume and having 0.4 and 0.6 of total blood flow to fat, respectively. The permeability-area cross products for the former compartment, which includes perirenal fat, were 0.05 and 0.025 L/h for Group I and II oligomers, respectively. For the latter fat compartment the cross products were 0.33 and 1.0 L/h for Group I and II oligomers, respectively.

CTFE elimination from the kidney was described as a first-order process that was determined by optimization against data on the urinary concentration of CTFE. A single, first-order rate constant for the metabolism of Group I and II oligomers was estimated by optimization against data on the rate of fluoride excretion in urine (Vinegar et al., 1989). It was assumed that 1.5 mol of fluoride were produced for each mole of CTFE metabolized. Fluoride release from bone was described by a first-order rate constant determined from data on urinary fluoride collected 20- to 60-days postexposure. Identical first-order constants were used to describe deposition in bone and excretion in urine. Kinetic constants and parameters used in the model are summarized in Table 3.1-1.



**Figure 3.1-1. Physiologically Based Pharmacokinetic Model for CTFE Oligomer Groups I and II.**  
 Abbreviations: CA = arterial concentration, CEX = exhaled concentration, CI = inhaled concentration, CV = venous concentration, CVF = venous fat concentration, CVK = venous kidney concentration, CVL = venous liver concentration, CVPR = venous perirenal fat concentration, CVR = venous rapidly perfused concentration, CVS = venous slowly perfused concentration, QC = cardiac output, QF = fat blood flow, QK = kidney blood flow, QL = liver blood flow, QP = alveolar ventilation, QPR = perirenal fat blood flow, QR = rapidly perfused blood flow, QS = slowly perfused blood flow, PAF = fat diffusional flow, PAL = liver diffusional flow, PAPR = perirenal fat diffusional flow, KEX = first-order excretion rate constant, KF = first-order metabolic rate constant, K0 = first-order fluoride from bone rate constant, K1 = first-order fluoride to bone and urine rate constants.

**TABLE 3.1-1. KINETIC CONSTANTS AND MODEL PARAMETERS USED IN THE PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR CHLOROTRIFLUOROETHYLENE OLIGOMERS**

Tissues	Volume (Fraction of body weight)
Liver	0.04 (single exposure)
Kidney	0.018
Fat	0.042
Perirenal fat	0.028
Slowly perfused	0.21
Rapidly perfused	0.72 - Liver weight
<b>Flows (L/h)</b>	
Alveolar ventilation	$14.0 \times BW^{0.74}$
Cardiac output	$13.0 \times BW^{0.74}$
	(Fraction of cardiac output)
Liver	0.25
Kidney	0.22
Fat	0.054
Perirenal fat	0.036
Slowly perfused	0.15
Rapidly perfused	0.29
	<b>Trimer</b>
Fat diffusion	$0.33 \times QF$
Perirenal fat diffusion	$0.05 \times QPR$
Liver diffusion	$0.08 \times QL$
	<b>Tetramer</b>
	$1.0 \times QF$
	$0.025 \times QPR$
	$0.08 \times QL$
<b>Partition Coefficients</b>	
Blood/air	33.0 (45.0)*
Liver/blood	20.0
Kidney/blood	12.8
Fat/blood	387.0
Perirenal fat/blood	387.0
Slowly perfused/blood	2.25
Rapidly perfused/blood	12.0
	23.0 (92.0)*
	40.0
	7.2
	1100.0
	1100.0
	2.25
	12.0
<b>Metabolism and Clearance (h<sup>-1</sup>)</b>	
KF	1.2/BW <sup>0.3</sup>
K0	0.0016/BW <sup>0.3</sup>
K1	0.82/BW <sup>0.3</sup>
KEX	0.000417/BW <sup>0.3</sup>

\* See discussion for explanation of double values.

**Abbreviations**

QF = fat blood flow  
QPR = perirenal fat blood flow  
QL = liver blood flow

KF = first-order metabolic rate constant  
K0 = first-order fluoride from bone rate constant  
K1 = first-order fluoride to bone and urine rate constants  
KEX = first-order excretion rate constant

Successful simulation of all data sets only could be accomplished by allowing the blood/air partition coefficient to vary between acute and subchronic exposures. Conceivably, tissues may have been altered pathologically so that partitioning characteristics had been changed. The blood/air partition coefficients for trimer and tetramer were 33 and 23, respectively, for the single exposure to 0.25 mg CTFE/L and the multiple exposure to 0.01 mg CTFE/L. However, these coefficients had to be increased to 45 and 92, respectively, to allow simulation of the multiple exposures to 0.25 mg CTFE/L. Hepatic effects were noted only at the higher concentration multiple exposure. Furthermore, the tetramer has been demonstrated to be more effective than the trimer in eliciting the hepatic response (Section 3.8, LD<sub>50</sub> and LD<sub>10</sub> Oral Toxicity Studies of Chlorotrifluororethylene Acids in F-344 Rats). The increase of the blood/air partition coefficient not only was required for those exposures that produced the hepatic lesions but a greater increase was necessary for the tetramer, which was more effective at producing the lesion. At this time, it is not clear how this correlation between hepatic lesion and increased blood/air partition coefficients can be explained.

#### RESULTS AND MODEL VALIDATION

##### *Single 6-h Inhalation Exposure to 0.25 mg CTFE/L*

For Groups I and II (Figures 3.1-2A and B) the fat concentration appeared to increase for at least one week postexposure with little actual change from 1 to 14 days postexposure. The peak fat concentrations measured were 25 and 10 mg/L for Group I and II oligomers, respectively. Concentrations of both Group I and II oligomers in liver and blood decreased rapidly for one day postexposure and more slowly thereafter.

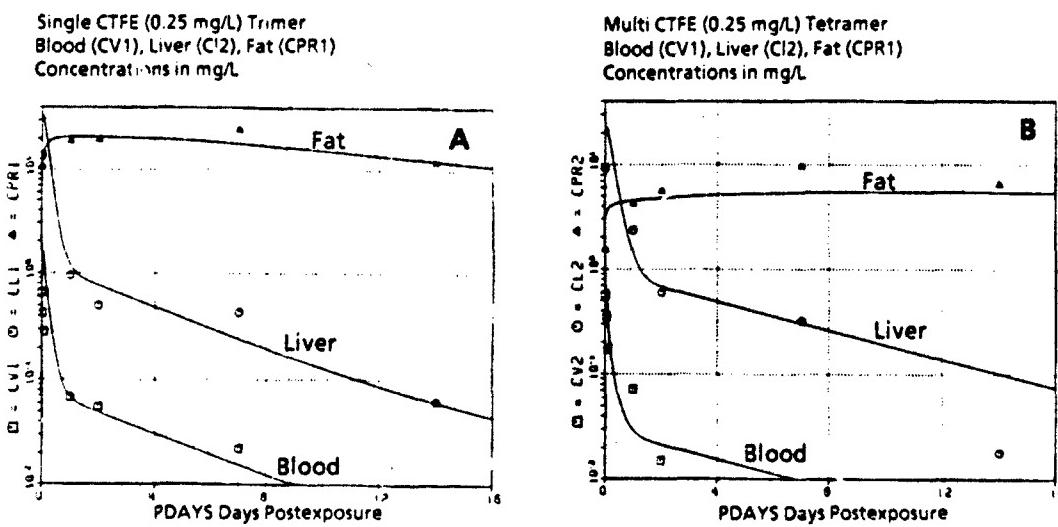


Figure 3.1-2. Male Fischer 344 Rat Blood, Liver, and Fat Concentrations of (A) Group I Oligomers (Trimer) and (B) Group II Oligomers (Tetramer) After a Single 6-h Exposure to 0.25 mg/L. Solid lines represent simulated results.

### 13-Week Inhalation Exposure, 6-h/Day, 5 Days/Week to 0.01 mg CTFE/L

Fat concentrations were 8 mg/L immediately and at two days after exposures were terminated, and the concentrations decreased to 4.5 mg/L at two weeks postexposure for Group I oligomers (Figure 3.1-3A) but remained at about 10 mg/L for the entire two-week period for Group II oligomers (Figure 3.1-3B). Blood concentrations were 0.032 mg/L immediately and 0.012 mg/L at two days postexposure for Group I oligomers, and 0.047 immediately, 0.031 mg/L at one hour postexposure, and undetectable thereafter for Group II oligomers, except for one individual with an apparent concentration of 0.022 mg/L at two days postexposure. Liver concentrations were 0.44 mg/L immediately, 0.14 at two days, and 0.03 at two weeks postexposure for Group I oligomers; and 0.7 mg/L immediately, 0.2 at two days, and 0.04 at two weeks for Group II oligomers.

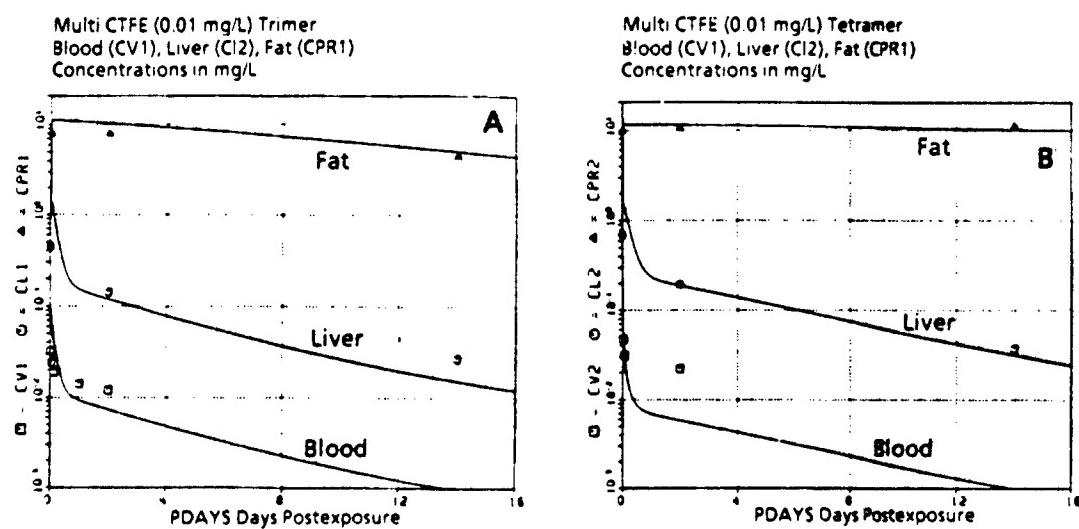


Figure 3.1-3. Male Fischer 344 Rat Blood, Liver, and Fat Concentrations of (A) Group I Oligomers (Trimer) and (B) Group II Oligomers (Tetramer) After 13 Weeks, 6 h/Day, 5 Days/Week Exposure to 0.01 mg/L. Solid line represents simulation.

### 13-Week Inhalation Exposure, 6-h/Day, 5 Days/Week to 0.25 mg CTFE/L

The fat concentration of Group I oligomers (Figure 3.1-4A) decreased continually so that at 63 days postexposure it was 9 mg/L compared with 296 mg/L immediately after the last exposure. Group II oligomer (Figure 3.1-4B) concentration in fat continued to increase slightly to 1065 mg/L for a week after the last exposure, and then decreased slowly to 279 mg/L at 63 days postexposure. The blood concentration of Group I oligomers was 0.9 mg/L immediately after the last exposure, 0.4 at one day postexposure, 0.025 at two weeks postexposure, and not detectable thereafter. The blood concentration of Group II oligomers was 2.1 mg/L immediately after the last exposure, 0.64 at one day postexposure, and detectable in one rat at 63 days (0.03 mg/L) postexposure. Liver concentrations of

Group I oligomers were 20 mg/L immediately postexposure, 5 at two days postexposure, and 0.043 at 63 days postexposure. Liver concentrations of Group II oligomers were 54 mg/L immediately postexposure, 21 at two days postexposure, and 1.1 at 63 days postexposure.

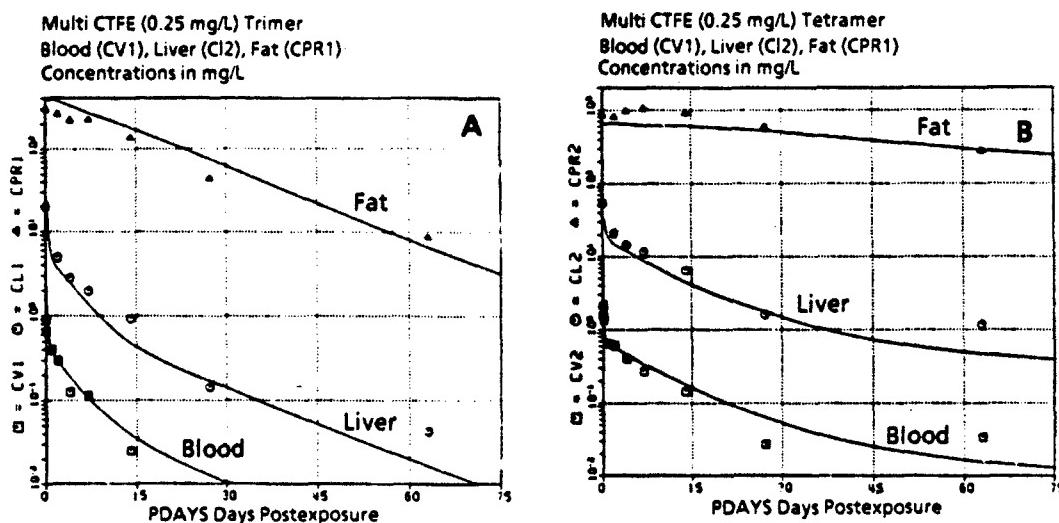
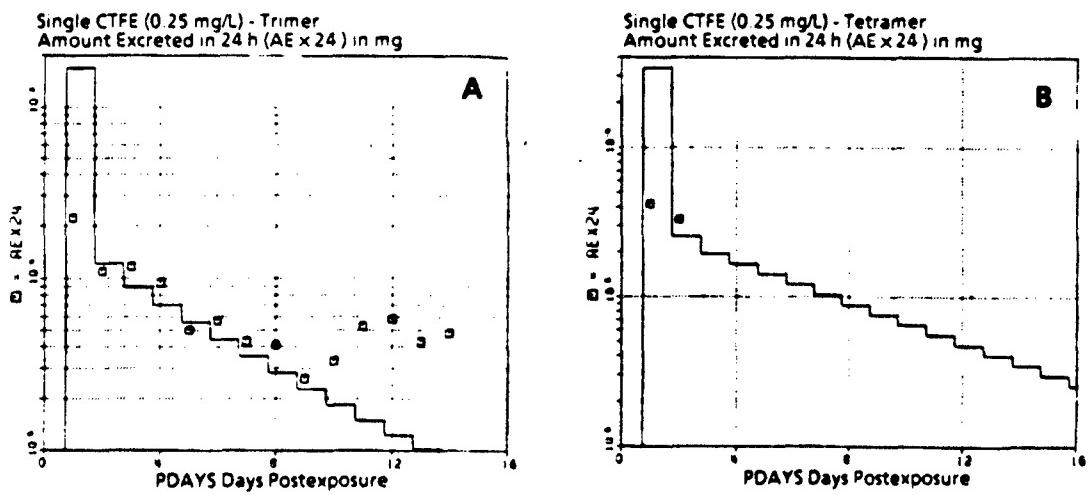


Figure 3.1-4. Male Fischer 344 Rat Blood, Liver, and Fat Concentrations of (A) Group I Oligomers (Trimer) and (B) Group II Oligomers (Tetramer) After 13 Weeks, 6 h/Day, 5 Days/Week Exposure to 0.25 mg/L. Solid line represents simulation.

#### Excretion of CTFE

Urine samples were collected for two weeks after single inhalation exposures to 0.25 mg/L. After the single exposure to 0.25 mg/L, the amount of Group I oligomers (Figure 3.1-5A) in the urine decreased from 0.00022 mg/24 h for the first day postexposure to 0.000026 mg/24 h for the ninth day postexposure. The amount excreted per day increased over the next five days to about 0.00005 mg/day. Group II oligomers (Figure 3.1-5B) were excreted at 0.000042 and 0.000033 mg/24 h for the first and second days postexposure, respectively, and were undetectable thereafter.



**Figure 3.1-5.** Male Fischer 344 Rat Urine Concentrations per 24 h of (A) Group I Oligomers (Trimer) and (B) Group II Oligomers (Tetramer) After a Single 6-h Exposure to 0.25 mg/L. Solid line represents simulation.

#### DISCUSSION

A difference in the kinetics of the trimer and tetramer can be seen by comparing the postexposure concentrations after single exposure to 0.25 mg CTFE/L (Figure 3.1-2) with the concentrations after multiple exposure (Figure 3.1-4). After a single exposure, concentrations of trimer in blood, liver, and fat were slightly higher than those of the tetramer, which reflects the 55:45 ratio of trimer to tetramer in the exposure atmosphere. However, the concentrations of tetramer were higher than those of the trimer after the multiple-exposure regimen. Furthermore, the concentration of tetramer in fat decreased at a slower rate than that of the trimer. Thus, fat served as the source of material to maintain higher concentrations in the liver where, at 63 days postexposure, the concentration of tetramer was more than an order of magnitude greater than that of the trimer.

Feces and urine were collected to account for elimination of CTFE from the body. Any CTFE that may have been present in fecal samples was below the limits of detection, although it is plausible that CTFE in feces had undergone bacterial degradation. However, CTFE was readily quantitated in urine samples. After single exposure (Figure 3.1-5) to 0.25 mg CTFE/L, Group II oligomers were detectable for two days postexposure but not thereafter. Group I oligomers were detectable for the full two weeks postexposure that urine samples were collected. Note that the initial amounts excreted for the 24 h immediately after exposure were 220 ng and 42 ng for Group I and II oligomers, respectively. Blood concentrations for Group I and II oligomers were 0.662 and 0.566 mg/L at 7 min,

0.427 and 0.349 mg/L at 1 h, and 0.068 and 0.072 mg/L at one day postexposure, respectively (Figure 3.1-2). This suggests that Group I oligomers with a molecular weight of about 425 were eliminated more readily by renal excretion than Group II oligomers with a molecular weight of about 520. The importance of molecular size as a determinant of whether a compound is more likely to be eliminated by urinary or biliary excretion has been reviewed (Calabrese, 1983; Withey, 1985). The molecular weight threshold for rats is around  $325 \pm 50$  with higher molecular weight compounds progressively being subject to biliary rather than urinary elimination.

Following 90-day inhalation exposure to 0, 0.25, 0.50, and 1.00 mg CTFE/L, liver weight to body weight ratios were 2.83, 5.22, 6.61, and 8.87 in male rats and 2.64, 3.06, 3.52, and 4.67 in female rats, respectively (Kinkead et al., 1989). Following 90-day inhalation exposure of male rats to 0, 0.01, 0.05, and 0.25 mg CTFE/L, liver weight/body weight ratios were 2.98, 2.80, 3.42, and 6.77, and the mean numbers of peroxisomes in a 30,000  $\times$  field of liver tissue were 3.0, 5.1, 6.4, and 11.9 (Section 3.3, Subchronic Inhalation Toxicity Studies on 3.1 Oil at Concentrations of 250, 50, and 10 mg/m<sup>3</sup>). The average rates of peroxisomal  $\beta$ -oxidation measured in liver tissue taken from the 0-, 0.01-, and 0.25-mg CTFE/L exposures were 2.90, 6.47, and 28.51  $\mu\text{m}/\text{min/g}$  following 90 days of exposure.

Male F-344 rats received weekly oral doses of either the trimer or tetramer acids of CTFE in corn oil at 1.66, 3.31, or 6.62 mg/kg or 0.54, 1.08, or 2.16 mg/kg, respectively. Controls received corn oil only. After six months, the rates of peroxisomal oxidation for the trimer were 3.2, 2.3, and 3.0  $\mu\text{mol}/\text{min/g}$  liver, and the rates for the tetramer were 4.6, 5.3, and 13.3  $\mu\text{mol}/\text{min/g}$  liver, compared with 3.1  $\mu\text{mol}/\text{min/g}$  liver for the controls (Section 3.9, Repeated-Dose Gavage Studies on Chlorotrifluoroethylene Acids).

Structurally similar compounds have been shown to produce both hepatic enlargement and peroxisomal proliferation. F-344 rats have been shown to have increased liver mass after single intraperitoneal injections of perfluorodecanoic acid (Olson and Andersen, 1983; George and Andersen, 1986; Van Rafelghem et al., 1987). Furthermore, increased numbers of hepatic peroxisomes were observed (Van Rafelghem et al., 1987).

The pharmacokinetic data are consistent with the hypothesis that the toxicity of CTFE is related to the preferential accumulation of longer chain oligomers. More specifically, the toxicity appears to be related to the accumulation of the acid metabolites of the oligomers, with that of the tetramer being more effective in eliciting the observed hepatic toxicity. Preliminary results (unpublished data) indicate that the suspected acid metabolite is present in the liver and in urine. Further work on identification and quantitation will allow the formation of the acid to be quantitatively linked with the PB-PK model, which already reasonably simulates the pharmacokinetics of the parent CTFE oligomers. Dose surrogates can be chosen that produce a known hepatotoxicity, the model can be

scaled up to humans, and estimates can be made of exposure scenarios necessary to reach the surrogate doses. This, of course, assumes that humans are subject to the same hepatotoxic effects of exposure to CTFE oligomers that have been observed in rats.

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### **3.2 ANALYSIS OF CHLOROTRIFLUORETHYLENE OLIGOMERS IN TISSUE, BLOOD, AND URINE**

**D.L. Pollard, C.S. Seckel, and H.C. Higman**

#### **ABSTRACT**

A mixture of trimers and tetramers of chlorotrifluoroethylene (CTFE) has been proposed as a hydraulic fluid in the Advanced Tactical Fighter system. The material under consideration contained approximately equal amounts of trimers and tetramers of CTFE end capped with chlorine to produce a viscosity of 3.1 poise. Previous range-finding studies were conducted to gather preliminary concentration-response data. The current study was implemented to define a no-effect level and to gather pharmacokinetic data pertinent to predicting effects based on inhaled concentration. Methods were developed in the initial study and adapted to the current work to allow for the decrease in analyte concentrations at reduced exposure levels. Animal group samplings were defined to permit time-course evaluation of concentration and clearance phenomena. Time-course blood samples, liver and fat tissue, and urine samples were analyzed for the presence of parent CTFE materials. The results of the study indicate that the CTFE materials were depleted rapidly from blood, less rapidly from liver and urine, and was stored for long periods of time in fat. There was more trimer than tetramer excreted in urine and more tetramer than trimer stored in fat. These sets of analytical data were interpreted for use in pharmacokinetic predictive modeling assessments.

#### **INTRODUCTION**

The purpose of this study was to provide information on the amount of CTFE oligomers found in tissues of animals that were exposed by inhalation to different levels of CTFE in order to define a no-effect level. The amount of CTFE and the distribution of the trimer and tetramer oligomers of the material in tissue, blood, and urine over defined time intervals were the analytical end points. These data were collected to provide data points to be utilized in predictive modeling assessments.

CTFE oil is a mixture of oligomers containing trimers and tetramers of CTFE end capped with chlorine. Inhalation studies conducted over 90 days at levels of 1000, 500, and 250 mg/m<sup>3</sup> of CTFE indicated that the liver and kidneys were target organs of CTFE toxicity. An additional study was conducted at levels of 250, 50, and 10 mg/m<sup>3</sup> to determine if, in fact, a no-observable-effect level could be defined for the material. Additional experiments were incorporated to develop a data base for testing the physiologically based pharmacokinetic (PB-PK) model for CTFE.

Two sets of animals were exposed for 6 h per day during the five-day workweek for 84 and 85 days, respectively. The first group, exposed for 85 days, was designated as target-organ animals and was exposed at 250, 50, and 10 mg CTFE/m<sup>3</sup>. The second set of animals was designated as PB-PK

study animals and was dosed for 84 days at levels of 250 and 10 mg CTFE /m<sup>3</sup>. Control animal sets were maintained for both studies. An additional exposure for one 6-h time period was conducted at 250 mg/m<sup>3</sup> and was designated as single-exposure PB-PK test animals.

Blood, urine, and tissue samples were collected from rats at defined time points to provide a comprehensive time-course analysis amenable to predictive modeling interpretation. Animals were sacrificed at time intervals to provide tissue samples that would be reflective of biological changes over time.

Analysis for CTFE oligomers in these biological matrices was performed by extraction followed by gas chromatographic analysis using electron capture detectors (GC-ECDs) as a sensitive and selective technique for the perhalogenated materials.

## MATERIALS

### Test Agent

Halocarbon oils are saturated, low molecular weight polymers of CTFE produced by a controlled polymerization and end capped with chlorine. The general chemical formula for these materials is Cl(CF<sub>2</sub>CFCI)<sub>n</sub>Cl. Fractions of the reaction product are distilled in the manufacturing process to produce discrete cuts of oligomers. The test agent used in this study was composed primarily of CTFE materials in which n = 3 or 4. These CTFE materials are discussed in the text as trimeric and tetrameric oligomers.

CTFE was supplied by the Air Force in three 75-lb plastic containers obtained from Halocarbon Products, Inc., Hackensack, NJ. The labeling information supplied is as follows.

MLO-87-347  
Safetol 3.1  
Hydraulic Fluid  
Batch # 87-23  
8-10-87  
P.O. 8400561

### Quality Assurance of Test Materials

Samples from each of the three 75-lb containers were analyzed by GC-ECD to verify uniformity of the composition of the materials. Preliminary investigations were conducted to ensure that the response of the ECDs to CTFE oligomers was representative of the concentration of the individual oligomers by comparing the responses from ECD to responses generated from a thermoconductivity detector (TCD). Standards were prepared and run interspersed with analytical samples throughout the course of the study.

## **METHODS**

### **Conditions for Gas Chromatographic Analysis**

The GC conditions used in this study for the analysis of samples and standards are shown below.

Gas chromatograph	Varian 3700
Column	0.75 mm i.d. x 30 m SPB-1
Column temperature	70 °C initial programmed to 30 at a 2° rise/min
Injector temperature	150 °C
Detector	ECD
Detector range	10
Detector temperature	300 °C
Helium carrier flow	10.2 mL/min
Argon-methane makeup	24 mL/min

### **Autosampler Injection**

Samples were injected on the chromatograph using a Varian 8000 (Varian Instrument Group, Walnut Creek, CA) series autosampler in order to extend the working analysis time and to enhance the reproducibility of both the injection volumes and absolute retention time data. GC samples were placed in the autosampler racks and sequenced for analysis using the Nelson analytical system.

### **Gas Chromatographic Analysis and Data Handling**

Samples, standards, and blanks were analyzed using the conditions listed previously. The raw chromatographic data were stored in the Nelson system and were integrated automatically. The results were stored on the Nelson computer and transferred electronically to a DEC VAX (Digital Equipment Corporation, Maynard, MA) mainframe computer for processing using RS/1 software. Standard curves were generated from standards data for each sample set using the RS/1 program. Concentrations of analytes were determined from these standard curves.

### **Sampling and Extraction Procedures**

**Blood Sampling.** Blood samples were obtained from the animals by tail vein bleeding using a 75-µL capillary tube attached to a sampling needle. The blood was pipetted immediately into 1.0 mL of hexane. The blood samples were extracted by mixing the sample and hexane for 16 h on an Evapotec® (Haake Buchler Instruments, Inc., Saddlebrook, NY) mixer. The samples then were centrifuged at 4 °C for 10 min at 2500 rpm. The hexane layers containing the analyte of interest then were transferred to 2-mL autosampler vials and stored at -70 °C until the time of analysis.

**Urine Sampling.** CTFE-exposed animals and control animals were placed in metabolism cages for collection of urine and feces samples. The total daily urine outputs were weighed and converted to volume using a density of 1.06 mg/mL. One milliliter of urine was pipetted from each sample into

2.0 mL of hexane and extracted by shaking on the Evapotec® mixer for 16 h. The resulting mixture was centrifuged at 4 °C for 10 min at 2500 rpm. The hexane layer was pipetted into a 2-mL autosampler vial and stored at -70 °C until the time of analysis.

**Tissue Sampling.** Tissue samples were removed at sacrifice according to a protocol designed to minimize cross contamination. A sacrifice of two PB-PK groups of animals was conducted at the midpoint of the repeat exposure (at 45 days exposure time) to determine CTFE concentration levels in the organs of those rats exposed to the 250 and 10 mg CTFE/m<sup>3</sup>. Four control animals also were sacrificed for tissue harvesting. Tissues removed for analysis from this midpoint sacrifice were lungs, liver, right kidney, perirenal fat, right testicle, muscle, and brain. Animals sacrificed at the conclusion of the inhalation study were sampled for perirenal fat and liver only based on assessments of the 45-day sacrifice. Additional samples of fat from different areas of the body were taken to determine the overall distribution of CTFE in adipose tissue.

A 1-g portion of tissue samples was placed in 20-mL scintillation vials containing 10 mL of hexane. The tissues were homogenized in the hexane using a Tissue-Mizer® (Tekmar, Cincinnati, OH). The resulting mixture was extracted for 16 h using the Evapotec® mixer. The homogenates then were centrifuged at 4 °C for 10 min at 2500 rpm. The hexane layers were pipetted into 20-mL scintillation vials and stored at -70 °C until the time of analysis.

#### **RESULTS AND DISCUSSION**

The chromatograms obtained from both standards and sample extracts contained two discrete groups of peaks. The first group was composed of five discrete peaks with retention times between 10 and 13.5 min. The second group contained five peaks with retention times between 22 and 28 min. These corresponded to the trimer and tetramer oligomers of CTFE, respectively. In the starting material these two groups of peaks were approximately equal in total area.

Calibration curves were obtained from concentration-response data developed from standard solutions over a working range of 1 to 500 ng/mL (1 to 500 ppb). The response of ECDs over a broad concentration range is not linear. The best-fit calibration curves established for this study were second-order (quadratic) equations. Concentrations of analytes were determined by comparison to standard curves using the RS/1 program software.

#### ***Mid-Term Sacrifice of Repeated Inhalation-Exposure Animals***

Two sets of animals were exposed to CTFE oligomers for 84 days at levels of 250 and 10 mg CTFE/m<sup>3</sup> for use in gathering data for pharmacokinetic studies. At 45 days a sacrifice was conducted of three animals from each set plus a set of control animals to gather preliminary data as to the concentration and distribution of the trimer and tetramer oligomers in organs and blood. Blood

samples were taken immediately after the animals were removed from the exposure chambers, and the animals were sacrificed immediately after the blood sampling. The tissue samples were treated as described in the Methods section of this report. Fat samples were dissolved in the hexane solvent and the data pertinent to fats were factored for the increase in volume associated with the dissolution of the fat in the solvent.

Average concentrations of CTFE oligomers in blood and tissue samples from animals sacrificed at 45 days into the 84-day exposure are shown in Table 3.2-1. Values are given in nanograms per milliliter of blood or grams of tissue.

TABLE 3.2-1. DISTRIBUTION OF CTFE OLIGOMERS IN BLOOD AND TISSUE FROM 45-DAY SACRIFICE

Tissue	Group I Oligomers (ng/g)	Group II Oligomers (ng/g)	Group I Oligomers (%)	Group II Oligomers (%)
<b>CTFE</b>				
Standard	-	-	55%	45%
<b>10-mg/m<sup>3</sup> Exposure Animals</b>				
Blood <sup>a</sup>	31	55	36	64
Lung	966	2022	32	68
Liver	73	158	32	68
Kidney	468	343	58	42
Fat <sup>b</sup>	6062	6306	49	51
Muscle	152	225	40	60
Brain	171	110	61	39
Testes	167	129	56	44
<b>250-mg/m<sup>3</sup> Exposure Animals</b>				
Blood <sup>a</sup>	1189	2230	27	73
Lung	16247	46377	26	74
Liver	7531	15175	33	67
Kidney	17697	19504	48	52
Fat <sup>b</sup>	229191	390918	37	63
Muscle	14941	32304	31	69
Brain	3553	4509	44	56
Testes	2682	3360	44	56

<sup>a</sup> Nanograms per milliliter.

<sup>b</sup> Fat samples taken from perirenal area.

It is interesting to note that the concentrations in fat are at least 10 times those of any other tissue and that, with the exception of the kidney and brain, the distribution of the oligomeric sets shows that a greater percentage of Group II oligomers were present in the tissue samples than were present in the original CTFE material.

Assessment of the data from the 45-day midterm sacrifice led to a decision to harvest only liver and perirenal fat samples from the full-term exposure animals and from a set of animals exposed for one 6-h inhalation period to 250 mg CTFE/m<sup>3</sup>.

#### **Full-Term Exposure and Single-Exposure Results**

The average CTFE oligomer concentration in the blood of animals exposed at the 250-mg/m<sup>3</sup> level for one day are compared to those exposed for 84 days (Table 3.2-2). The data presented are average postexposure values for sets of four to eight animals sampled at the designated time points. The single-exposure animals were sampled at time intervals through 14 days postexposure. The animals exposed for 84 days were sampled through 252 days. A time for sampling of 0 denotes samples taken immediately after removal from the exposure chambers.

**TABLE 3.2-2. COMPARISON OF BLOOD LEVELS OF CTFE FROM 250-mg/m<sup>3</sup> EXPOSURES**

Time of Sampling	Single Exposure		Repeat Exposure	
	Group I Oligomers (ng/mL)	Group II Oligomers (ng/mL)	Group I Oligomers (ng/mL)	Group II Oligomers (ng/mL)
0	662	566	914	1578
1 h	429	349	888	2158
2 h	313	170	—	—
3 h	—	—	648	1367
1 day	69	76	396	641
2 days	53	19	292	597
4 days	—	—	124	406
7 days	22	0	113	275
14 days	0	0	25	143
28 days			0	28
63 days			0	13
126 days			0	0
252 days			0	0

A set of animals exposed to 10 mg/m<sup>3</sup> of CTFE oligomers were sampled also. The 10-mg/m<sup>3</sup> repeat-exposure data are not presented in tabular form. The concentrations were 30- to 50-fold less than the 250-mg/m<sup>3</sup> sets, and the measurable oligomers were depleted in less than two days.

These data indicate that the single-exposure animals had greater percentages of Group I oligomers in the blood and that levels in the single-exposure animals were depleted in less than 14 days. The repeat-exposure animal set indicated relatively greater amounts of Group II oligomers throughout the course of the study, and detectable levels of CTFE remained in the blood for a full three months after exposure.

Average values of CTFE oligomers in urine collected from the single- and repeat-exposure 250-mg CTFE/m<sup>3</sup> animal sets over the same period as the blood sampling are shown in Table 3.2-3.

Urine samples were collected daily from both sets of animals for two weeks after removal from the exposure chambers. These data are averages of four animals per group. The animals used for the sampling were those held for the full term of the study. The urine data showed a much greater percentage of Group I oligomers than were present in the standard exposure material. The relative levels of Group II oligomers were much less of a percentage of excreted CTFE material than the levels present in the standard. The levels of CTFE Group I materials were much higher in the repeat-exposure animals and were more persistent. The levels of Group II oligomers dropped rapidly to below detectable limits. In the single-exposure animals, no Group II oligomers were detected after Day 2 postexposure. The repeat set had trace levels of Group II oligomers through 10 days postexposure.

TABLE 3.2-3. COMPARISON OF URINE LEVELS OF CTFE FROM 250-mg/m<sup>3</sup> EXPOSURE

Days Postexposure	Single Exposure		Repeat Exposure	
	Group I Oligomers (ng/mL)	Group II Oligomers (ng/mL)	Group I Oligomers (ng/mL)	Group II Oligomers (ng/mL)
1	221	32	453	138
2	110	17	570	96
3	116	0	508	134
4	93	0	391	90
5	50	0	303	69
6	57	0	321	27
7	45	0	265	39
8	40	0	186	47
9	26	0	205	21
10	34	0	177	11
11	53	0	129	0
12	57	0	152	0
13	42	0	131	0
14	48	0	111	0

Concentrations of CTFE oligomers in liver tissue from the animals exposed to 250 mg CTFE/m<sup>3</sup> for one day and for 85 days are presented in Table 3.2-4. The time postexposure means that animals sacrificed at Day 0 were sacrificed immediately after being sampled for blood analysis. Average values are presented for groups of four animals sacrificed per set. Control animals gave 0 values in almost all instances. Data also are presented for the repeat-exposure animals exposed at 10 mg CTFE/m<sup>3</sup>.

The single-exposure animal sets showed less material than did the repeat-exposure sets. Also, the repeat-exposure animals at the 250-mg/m<sup>3</sup> level showed a significant increase in retention of the Group II oligomers as compared to the starting material. Significant levels of CTFE material were still

found in the livers of repeat-exposure animals after 252 days postexposure, whereas the concentration of CTFE oligomers dropped off rapidly in the single-exposure, 250-mg/m<sup>3</sup> sets.

TABLE 3.2-4. COMPARISON OF CTFE LEVELS IN LIVER\* FROM 250- AND 10-MG/M<sup>3</sup> EXPOSURES

Days Postexposure	Single Exposure		Repeat Exposure	
	Group I Oligomers (ng/g)	Group II Oligomers (ng/g)	Group I Oligomers (ng/g)	Group II Oligomers (ng/g)
<b>250-mg/m<sup>3</sup> Exposure</b>				
0*	13644	9233	19767	53792
1	923	2336	-	-
2	496	597	4893	20888
4	-	-	2775	14447
7	428	320	1956	11690
14	62	14	928	6468
28			141	1613
63			42	1167
126			16	1159
252			0	338
<b>10-mg/m<sup>3</sup> Repeat Exposure</b>				
0			442	695
2			141	195
14			27	37

\* Denotes sacrifice immediately after removal from exposure chambers.

The concentrations of CTFE found in the perirenal fat of animals exposed to 250 and 10 mg CTFE/m<sup>3</sup> for the full term of the inhalation study and for animals exposed to 250 mg/m<sup>3</sup> for one 6-h period are shown in Table 3.2-5. It is of interest to note that the single-exposure animals and the animals exposed to 10 mg/m<sup>3</sup> for the full inhalation study had much higher relative concentrations of the Group I oligomers over the time course of the observations than did the animals exposed to 250 mg/m<sup>3</sup>, where there was a definite preferential concentration of Group II oligomers from the first day of sacrifice throughout the period of observation. Even at 252 days postexposure there were significant levels of Group II oligomers present in the perirenal fat of the repeat 250-mg/m<sup>3</sup> exposure rats. At Day 63 of the sacrifice schedule, samples were collected from additional adipose tissue for comparison to the perirenal fat samples. The distribution of CTFE oligomers in mesenteric, inguinal, and outer abdominal adipose tissue was determined. The distribution and concentration of CTFE oligomers in these additional adipose tissues were similar to those found in perirenal fat and are not reported in tabular form.

TABLE 3.2-5. COMPARISON OF CTFE LEVELS IN FAT\* FROM 250- AND 10-mg/m<sup>3</sup> EXPOSURES

Days Postexposure	Single Exposure		Repeat Exposure	
	Group I Oligomers (ng/g)	Group II Oligomers (ng/g)	Group I Oligomers (ng/g)	Group II Oligomers (ng/g)
<b>250-mg/m<sup>3</sup> Exposure</b>				
0	13213	1844	362715	1046968
1	23171	5150	—	—
2	24889	6841	320603	996097
4	—	—	270011	1194376
7	30125	11731	275593	1303384
14	14791	7957	168364	1104300
28			52943	726375
63			8000	341112
126			600	123149
252			0	27113
<b>10-mg/m<sup>3</sup> Repeat Exposure</b>				
0			9766	12027
2			9718	13091
14			5534	13664

\* Denotes perirenal fat sample.

## CONCLUSIONS

Data are presented for the time course analysis of the CTFE concentration in blood, urine, and tissue samples of animals exposed by inhalation to CTFE oligomers. These data showed a rapid decrease in concentrations of CTFE in blood after removal from the inhalation chambers. Analysis of urine samples indicates a similar rapid decrease of CTFE material eliminated in the urine after the animals are removed from the inhalation chambers. Animals exposed for one 6-h period to 250 mg CTFE/m<sup>3</sup> and the animals exposed to 10 mg/m<sup>3</sup> for 84 days showed a rapid depletion of CTFE from liver tissue. Animals exposed repeatedly for 85 days showed a retention of CTFE oligomers in liver tissue with a preferential concentration of the Group II oligomers at detectable concentrations throughout the first 252 days of the sacrifice schedule. Perirenal fat and adipose tissue from mesenteric, outer abdominal cavity, and inguinal areas of the rat all showed a significant retention of CTFE oligomers with a preferential concentration of Group II oligomers over the first 252 days of the sacrifice schedule.

Data are presented as average values to illustrate trends in the analytical sets. These data were utilized to provide input into a PB-PK model which simulates the uptake, distribution, metabolism, and elimination of CTFE oligomers.

### **3.3 SUBCHRONIC INHALATION TOXICITY STUDIES ON 3.1 OIL AT CONCENTRATIONS OF 250, 50, AND 10 mg/m<sup>3</sup>**

**E.R. Kinkead, C.D. Flemming, and H.G. Wall**

#### **ABSTRACT**

Chlorotrifluoroethylene (CTFE) oligomer (3.1 oil) is a chlorofluorocarbon oil consisting of C6 and C8 oligomers. Physical properties such as high thermal stability, good lubricity, and high dielectric strength make CTFE promising for potential use in hydraulic fluid systems. A previous inhalation study indicated that the liver is the target organ of CTFE toxicity. Gross liver hypertrophy and microscopic hepatocytomegaly were the prime lesions noted. To determine a no-effect concentration, male Fischer 344 (F-344) rats were exposed to air only, and 10, 50, and 250 mg CTFE/m<sup>3</sup> for 13 weeks. Mean body weights of the highest concentration test group were slightly, but not significantly, depressed during the final two-thirds of the study. Alkaline phosphatase and blood urea nitrogen (BUN) values were increased in this group at sacrifice. Significant concentration-related increases in relative kidney and liver weights occurred in the 250- and 50-mg CTFE/m<sup>3</sup> groups. Hepatocytomegaly was a common finding in all rats exposed at these concentrations. Examination of hepatocytic ultrastructure revealed slightly swollen mitochondria in the two highest exposure groups. The number of peroxisomes per visual field was significantly greater in the highest exposure group only. The associated activity of peroxisomal beta oxidation of palmitoyl coenzyme A (CoA) also showed a concentration-dependent increase. A no-effect level of 10 mg CTFE/m<sup>3</sup> was identified based on light and electron microscopic, hematologic, and in-life data.

#### **INTRODUCTION**

CTFE oligomer is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectric strength. A recently concluded 90-day inhalation study to 1000, 500, and 250 mg CTFE/m<sup>3</sup> in this laboratory resulted in a dose-dependent depression in body weight gains of male rats (Kinkead et al., 1989). Alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) values examined at the conclusion of the study indicated a treatment-response effect in the male test rats but not in the female test rats. Relative kidney and liver weights of the male test rats were elevated significantly when compared to the respective control groups. The primary target organ of CTFE was the liver. Gross liver hypertrophy and microscopic hepatocytomegaly were the principal manifestations of CTFE-induced hepatotoxicity.

This multiconcentration inhalation study was conducted to provide a no-observable-effect level associated with repeated inhalation of CTFE for 90 days. This study included only male rats,

which appear to be more susceptible than female rats to CTFE vapors. Also included in the study were rats that were exposed at the highest concentration level and maintained one year postexposure to determine if treatment-related effects were reversible.

#### **MATERIALS AND METHODS**

##### ***Animals***

Upon receipt from Charles River Breeding Labs, Kingston, NY, 226 male F-344 rats, six weeks of age, were quality-control tested and found to be in acceptable health. The animals were randomized using a proprietary modular software system (PATH/TOX® System, Cedar Knolls, NJ) which assigned animals to groups. They were group housed (two to three per cage) in clear plastic cages with wood-chip bedding prior to the study. The rats (10 weeks of age at initial exposure) were housed individually and assigned to specific exposure cage locations during the study. The exposure cages were rotated in a clockwise manner (moving one position) within the 690-L inhalation chambers each exposure day. Water and feed (Purina Formulab #5008) were available ad libitum, except during the inhalation period when food was removed and 10 h prior to sacrifice when the rats were fasted. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

##### ***Test Agent***

The CTFE sample used in this study, which did not contain any additive, was supplied by the U.S. Air Force and obtained from Halocarbon Products, Co., Hackensack, NJ. Three plastic cans containing approximately 75 lb of hydraulic fluid were received. The labels on the containers identified the sample as shown below.

MLO-87-347  
Safetol® 3.1  
Hydraulic Fluid  
Batch #87-23  
8-10-87  
P.O. NO. 8400561

Chemically, halocarbon oils such as CTFE are saturated, low molecular weight polymers of chlorotrifluoroethylene having the general formula  $(CF_2CFCI)_n$ . They are made using a controlled polymerization technique and are stable, with the terminal groups being completely halogenated. The products then are separated by vacuum distillation into various fractions, from light oils to waxes.

## **INHALATION TOXICITY**

### **Exposure Regimen**

Male F-344 rats were exposed for 6 h/day, 5 days/week, for 13 weeks (65 exposures over a 90-day test period). Twenty-six animals were exposed to either air only, or 50 or 250 mg CTFE/m<sup>3</sup>. Eighteen animals were exposed to 10 mg/m<sup>3</sup>. Ten animals from each group were sacrificed at the conclusion of the exposure period, whereas the remaining rats were held for postexposure serial sacrifice (three rats every three months for control, 50-, and 250-mg/m<sup>3</sup> groups, and every six months for the 10-mg/m<sup>3</sup> group).

Two groups of male F-344 rats were included in the study for development of a pharmacokinetic model. The rats were exposed to either air alone, or 10 or 250 mg CTFE/m<sup>3</sup> on the same schedule as detailed above. Liver sections were removed from these animals for peroxisomal β-oxidation assays following 45 and 90 days of exposure and at 4, 14, 28, 63 and 126 days postexposure.

### **Animal Response Assessment**

Records were maintained for body weights (one day pre-exposure, weekly during the first four weeks, then biweekly thereafter), signs of toxicity, and mortality. Four rats per exposure group were removed midway through the study for a single weekend. Two 24-h urine samples were collected for inorganic fluoride analysis. This same group of rats was maintained in metabolism cages for 14 days following the 90-day study for similar urine fluoride analyses. Ten rats per group were sacrificed on the day following the final exposure. Euthanasia was accomplished via halothane inhalation overdose. At sacrifice, gross pathology was performed and tissues were harvested for histopathologic examination. Wet tissue weights were determined on adrenals, brain, heart, kidneys, liver, lungs, spleen, testes, and thymus. Tissues for histopathologic examination were fixed in 10% neutral-buffered formalin, trimmed, and processed further via routine methods for hematoxylin and eosin-stained paraffin-embedded sections (Luna, 1968).

Additionally, blood was drawn for hematology and clinical chemistry assays. Erythrocytes were enumerated on a Coulter counter (Coulter Electronics, Hialeah, FL), and sera for clinical chemistry evaluation were assayed on an Ektachem 700 XR (Eastman Kodak, Rochester, NY). Selected hematological parameters and absolute leukocyte differentials were determined according to established procedures. Sera were processed according to the procedures in the *Ektachem Operations Manual*.

Peroxisomal β-oxidation assays were performed on approximately 1 g of liver tissue. Samples were removed from the median lobe and placed in ice-cold 0.25M sucrose. The sample was

homogenized, then centrifuged at 1500 x g for 10 min. A portion of the resultant supernatant was used to measure the rate of palmitoyl CoA oxidation (Lazarow, 1982).

#### Statistical Analysis

Comparisons of mean body weights were performed using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A two-factorial analysis of variance with multivariate comparisons was used to analyze the histopathology, clinical chemistry, and organ weight data. The histopathology data were analyzed using one of the following nonparametric tests: Fisher's Exact Test, or, if not valid, Yates' Corrected Chi-Square (Zar, 1974). Peroxisomal  $\beta$ -oxidation assays were analyzed using a nonparametric analysis of variance (SAS, 1985). A probability of 0.05 inferred a significant change from controls.

#### RESULTS

Mean body weights of the 250-mg CTFE/m<sup>3</sup> group were slightly depressed over the last two-thirds of the study when compared to the other rat groups (Figure 3.3-1). However, the difference in mean weights was not statistically significant at any of the time points. Test rats maintained postexposure are gaining weight at a rate comparable to the control rats.

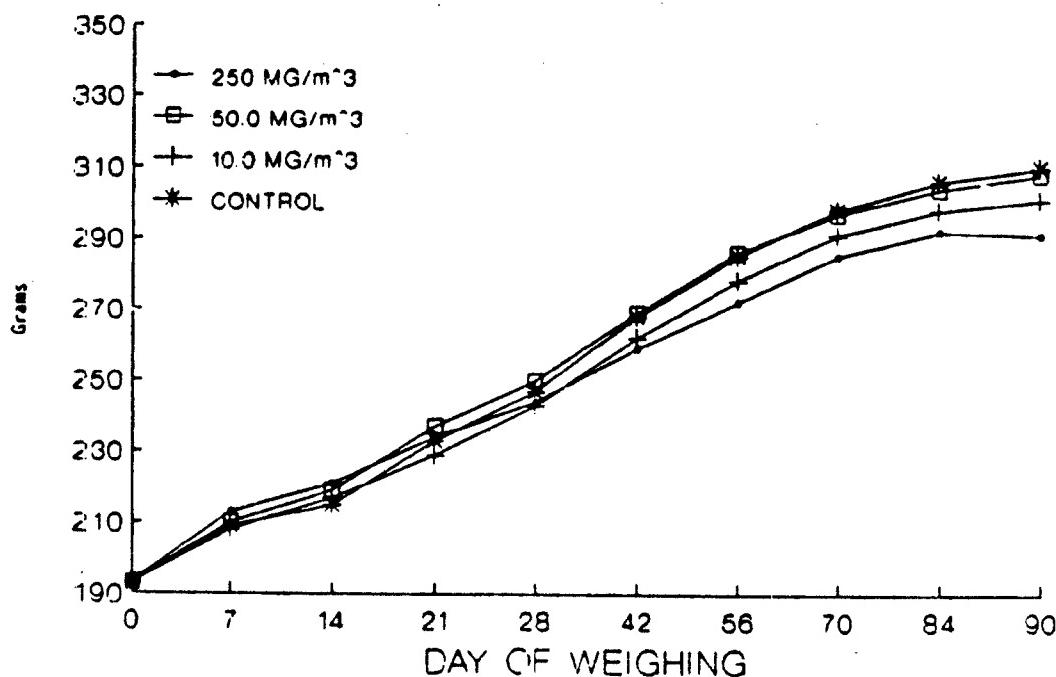


Figure 3.3-1. Effect of 90-Day CTFE Inhalation Exposures on Mean Body Weight of Male F-344 Rats.

Inorganic fluoride analyses of urine sampled midway through the 90-day study and again for 14 days following completion of the study are shown in Figure 3.3-2. An increase ( $p<0.01$ ) in total inorganic fluoride excreted in the urine of the 250-mg CTFE/m<sup>3</sup> rats was evident midway through the exposure and for the 14-day period postexposure. The increase ( $p<0.05$ ) in inorganic fluoride excreted in the urine of rats exposed to 50 mg CTFE/m<sup>3</sup> also had peaked at 45 days and continued through four days postexposure. No increase in inorganic fluoride was detected in the urine of rats exposed to 10 mg CTFE/m<sup>3</sup> at any of the sampling points in the study.

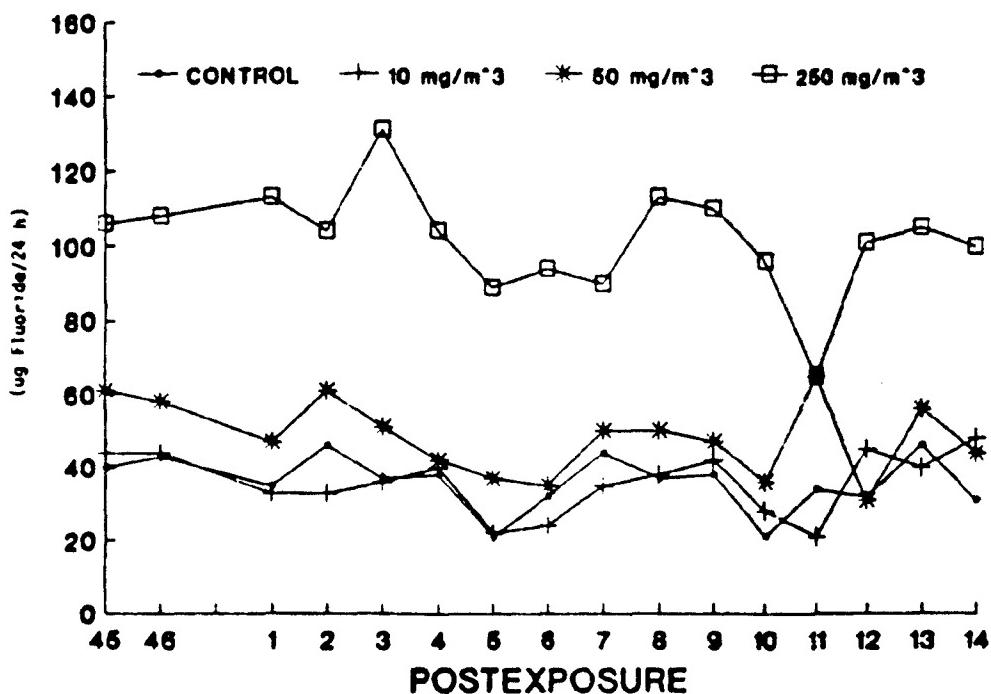


Figure 3.3-2. Total Inorganic Fluoride Excreted in the Urine of Male Rats (Micrograms of Fluoride/24 h) following Repeated Exposure to 250 mg CTFE/m<sup>3</sup>.

The mean alkaline phosphatase values for the high-concentration rat group were significantly different ( $p<0.01$ ) from controls. Increased ( $p<0.01$ ) BUN values also were observed in these animals. AST and ALT values appeared unaffected. Red blood cell count and hemoglobin and hematocrit values were depressed ( $p<0.01$ ) in the 250-mg CTFE/m<sup>3</sup> group. Mean corpuscular volume, mean corpuscular hemoglobin, and the platelet count were elevated significantly ( $p<0.01$ ) in the high-treatment group.

Significant concentration-related increases ( $p<0.01$ ) in relative kidney weights occurred in the 250- and 50-mg CTFE/m<sup>3</sup> groups (Table 3.3-1). Similarly, a concentration-related increase in relative liver weights occurred in the two higher treatment groups. Relative liver weights were increased over controls by 14 and 127% in the 50- and 250-mg CTFE/m<sup>3</sup> groups, respectively.

TABLE 3.3-1. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE

	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Kidney Ratio <sup>b</sup>	2.06 ± 0.06 0.69 ± 0.01	2.14 ± 0.08 0.74 ± 0.01	2.35 ± 0.61 0.78 ± 0.01 <sup>c</sup>	2.60 ± 0.05 <sup>c</sup> 0.93 ± 0.01 <sup>c</sup>
Heart Ratio	1.01 ± 0.03 0.34 ± 0.01	0.98 ± 0.03 0.34 ± 0.01	0.93 ± 0.02 0.31 ± 0.01	0.94 ± 0.03 0.34 ± 0.01
Brain Ratio	1.94 ± 0.03 0.65 ± 0.01	1.92 ± 0.03 0.68 ± 0.02	1.93 ± 0.02 0.64 ± 0.01	1.91 ± 0.01 0.68 ± 0.01
Liver Ratio	8.89 ± 0.27 2.98 ± 0.05	8.17 ± 0.37 2.80 ± 0.06	10.35 ± 0.36 3.42 ± 0.06 <sup>c</sup>	18.92 ± 0.33 <sup>c</sup> 6.77 ± 0.10 <sup>c</sup>
Spleen Ratio	0.65 ± 0.02 0.22 ± <0.01	0.62 ± 0.02 0.21 ± <0.01	0.63 ± 0.02 0.21 ± 0.01	0.58 ± 0.02 0.21 ± <0.01
Thymus Ratio	0.31 ± 0.03 0.10 ± 0.01	0.26 ± 0.03 0.09 ± 0.01	0.26 ± 0.02 0.09 ± 0.01	0.21 ± 0.02 0.08 ± 0.01
Lungs Ratio	1.55 ± 0.09 0.52 ± 0.03	1.73 ± 0.11 0.59 ± 0.02	1.68 ± 0.07 0.55 ± 0.02	1.52 ± 0.06 0.54 ± 0.02
Adrenals Ratio	0.08 ± 0.01 0.03 ± <0.01	0.06 ± 0.01 0.02 ± <0.01	0.09 ± 0.03 0.03 ± 0.01	0.07 ± 0.01 0.03 ± <0.01
Testes Ratio	3.11 ± 0.05 1.05 ± 0.02	3.09 ± 0.05 1.07 ± 0.02	3.12 ± 0.06 1.03 ± 0.02	3.14 ± 0.02 1.13 ± 0.02
Whole Body <sup>d</sup>	298.3 ± 6.3	290.1 ± 8.0	302.2 ± 6.5	279.8 ± 4.7

<sup>a</sup> Mean ± SEM, N = 10

<sup>b</sup> Organ weight/body weight × 100.

<sup>c</sup> Significantly different from control, p < 0.01, as determined by a one-factorial analysis of variance with multivariate comparisons.

<sup>d</sup> Fasted weights.

Gross pathologic findings at the conclusion of the 90-day exposure consisted of gross liver enlargement in the mid- and high-level CTFE-exposed rats, which subsequently was determined to be statistically significant when compared to controls (p < 0.01). Histologically, the prevalence of hepatocytomegaly was 100% in the 250- and 50-mg CTFE/m<sup>3</sup>-exposed rats (Table 3.3-2). Both groups also had hypergranular, eosinophilic cytoplasm and loss of basophilic stippling (in the cytoplasm of hepatocytes). The latter lesion occurred at a lesser degree of severity in the 50-mg CTFE/m<sup>3</sup> group rats. Forty percent of the rats in the high treatment group had cytoplasmic vacuolization.

The hepatocellular cytomegaly noted in the 50-mg CTFE/m<sup>3</sup> group was less pronounced. The cells were slightly swollen with rounded edges and indistinct borders. Also, the volume of cytoplasm, hypergranularity, and eosinophilia was less severe than the 250-mg CTFE/m<sup>3</sup> group. The loss of basophilic stippling in the cytoplasm of the hepatocytes was not a total loss, as was the case in the high-exposure group.

**TABLE 3.3-2. INCIDENCE (%) SUMMARY OF SELECTED MICROSCOPIC LESIONS OF MALE RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CTFE**

Organ/Lesion	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
<b>Liver</b>				
Hepatocellular cytomegaly	0	0	100 <sup>a</sup>	100 <sup>a</sup>
Eosinophilic hypergranular cytoplasm	0	0	100 <sup>a</sup>	100 <sup>a</sup>
Loss of basophilic stippling <sup>b</sup>	0	0	100 <sup>a</sup>	100 <sup>a</sup>
Cytoplasmic vacuolization	0	0		
<b>Kidneys</b>				
Hyaline droplets (severity) <sup>c</sup>	100 1.0	100 1.0	100 1.2	100 3.0 <sup>d</sup>
Laminated concretions	100	100	100	100

<sup>a</sup> Significantly different from control, p<0.01, as determined by Chi-Square test.

<sup>b</sup> Refers to cytoplasm of hepatocytes.

<sup>c</sup> Grades of severity based on 0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe, and 5 = necrotic.

<sup>d</sup> Significantly different from control, p<0.05, as determined by Chi-Square test.

The low-exposure group (10 mg CTFE/m<sup>3</sup>) had no detectable lesions within the hepatocytes. Cells were of normal size, shape, and tinctorial quality and could not be distinguished from the hepatocytes of the control animals.

Microscopic examination of the kidneys revealed the presence of eosinophilic hyaline droplets within the cytoplasm of the epithelial cells of the proximal convoluted tubules. This lesion was present in all animals; however, the hyaline droplets were larger and of greater number in the 250 mg CTFE/m<sup>3</sup>-exposed group. A greater degree of severity (moderate compared to minimal in the other three groups) and distribution (much more numerous and present in many more tubules) was observed in the hyaline droplet formation in the high-exposure group. The only other kidney lesion was laminated concretions, which were anchored on the basement membrane of many proximal convoluted tubules. The laminated concretions were distributed equally throughout all rat groups and appeared to be inert.

Morphometric examination of hepatocytic ultrastructure in livers from control and CTFE-exposed rats determined that the nuclei appeared normal in control rat hepatocytes. There was a slight increase in the degree of margination of the chromatin in the 50- and 250-mg CTFE/m<sup>3</sup> groups. Although there was a slight shrinkage of the nucleus in occasional cells from the controls, 10-, and 50-mg CTFE/m<sup>3</sup> groups, the shrinkage of the nucleus occurred in more liver cells of animals exposed to the highest concentration of CTFE.

No differences were noted in the mitochondria of the 10-mg CTFE/m<sup>3</sup> group when compared to the controls. Mitochondria were normal to slightly swollen in the 50-mg CTFE/m<sup>3</sup> group. The degree of swelling in mitochondria from rats exposed to the highest concentration of CTFE ranged

from normal to slightly swollen. Swelling progressed from intracristal dilatation to cristal displacement. No difference occurred in the amounts of smooth endoplasmic reticulum (SER) in hepatocytes of rats exposed at 10 mg CTFE/m<sup>3</sup> when compared to the control rats. SER increased slightly above control levels in the 50-mg CTFE/m<sup>3</sup> group and appeared to be even greater for the 250-mg CTFE/m<sup>3</sup> group. Only in the high-exposure group did the SER appearance begin to change from an interconnecting network of membranes seen in control liver to individual vesicles, which also was noted at the higher doses in the first 90-day study (Kinkead et al., 1989).

Although the number of peroxisomes per visual field increased with exposure, the increase in peroxisomes was statistically significantly different from the control value only at the highest treatment group (Table 3.3-3). The rough endoplasmic reticulum (RER) appeared normal in hepatocytes examined from the two lower exposure groups. In the 10-mg CTFE/m<sup>3</sup> group, some RER appeared to be degranulated. Animals from the 50-mg CTFE/m<sup>3</sup> concentration group showed some RER degeneration, and some of the stacks of RER looked smaller or not as tightly packed as in controls. Hepatocytes from rats exposed at 250 mg CTFE/m<sup>3</sup> had loss of stacks and smaller stacks that were less tightly packed whereas more RER appeared to be breaking down into SER. There was a partial loss of ribosomes on occasional strands of RER. Occasional membranous whorls were seen in the cytoplasm of hepatocytes from all animals in the 50- and 250-mg CTFE/m<sup>3</sup> groups. The amount of lipid in hepatocytes increased after exposure to CTFE. The changes were not concentration dependent because the 10- and 250-mg CTFE/m<sup>3</sup> groups were the same and the 50-mg CTFE/m<sup>3</sup> group was higher than control amounts but lower than the other test groups.

TABLE 3.3-3. MORPHOMETRIC EVALUATION OF HEPATOCYTE ULTRASTRUCTURE IN MALE F-344 RATS FOLLOWING 90-DAY REPEATED INHALATION EXPOSURE TO CTFE

	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Peroxisomes <sup>a</sup>	3.0 ± 0.4	5.1 ± 0.7	6.4 ± 1.1	11.9 ± 1.3 <sup>b</sup>
Number of animals	11	15	14	14

<sup>a</sup> Mean ± SEM of the number of peroxisomes in a 30,000× field using an 8 × 10 photograph (9 to 20 photographs per animal).

<sup>b</sup> Significantly different than control ( $p < 0.01$ ) as determined by a two-factorial analysis with multivariate comparisons.

Blood chemistry and hematology data collected from rats sacrificed postexposure revealed that the mean alkaline phosphatase values for the 250-mg CTFE/m<sup>3</sup> group (144 U/L) were no longer significantly ( $p < 0.01$ ) higher than the control group (136 U/L). BUN values of the highest exposed group remained higher than any other treatment group; however, the difference was statistically significant only at six-months postexposure (15.2 mg/dL versus 18.7 mg/dL).

Significant concentration-related increases ( $p < 0.01$ ) in relative liver and kidney weights were still evident at three months postexposure (Table 3.3-4). Increases of 10 and 4% were evident in relative weights of livers and kidneys, respectively, of rats exposed to 250 mg CTFE/m<sup>3</sup>. These differences were resolved by the six-month postexposure sacrifice.

**TABLE 3.3-4. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE F-344 RATS EXAMINED THREE AND SIX MONTHS POSTEXPOSURE**

Organ	Control	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Three Months Postexposure			
Kidney Ratio <sup>b</sup>	2.33 ± 0.08 0.68 ± 0.01	2.37 ± 0.07 0.65 ± 0.01	2.30 ± 0.0 0.71 ± 0.02 <sup>c</sup>
Liver Ratio	9.29 ± 0.22 2.50 ± 0.02	9.38 ± 0.28 2.59 ± 0.03	10.30 ± 0.49 3.16 ± 0.11 <sup>c</sup>
Whole Body <sup>d</sup>	371.4 ± 6.7	362.6 ± 7.1	329.9 ± 8.0 <sup>c</sup>
Six Months Postexposure			
Kidney Ratio	2.50 ± 0.02 0.59 ± 0.05	2.36 ± 0.1 0.59 ± 0.05	2.66 ± 0.07 0.66 ± 0.02
Liver Ratio	11.56 ± 0.22 2.73 ± 0.06	10.43 ± 0.44 2.61 ± 0.02	11.06 ± 0.13 2.76 ± 0.04
Whole Body <sup>d</sup>	298.3 ± 6.3	302.2 ± 6.5	279.8 ± 4.7

<sup>a</sup> Mean ± SEM, N = 4.

<sup>b</sup> Organ weight/body weight × 100.

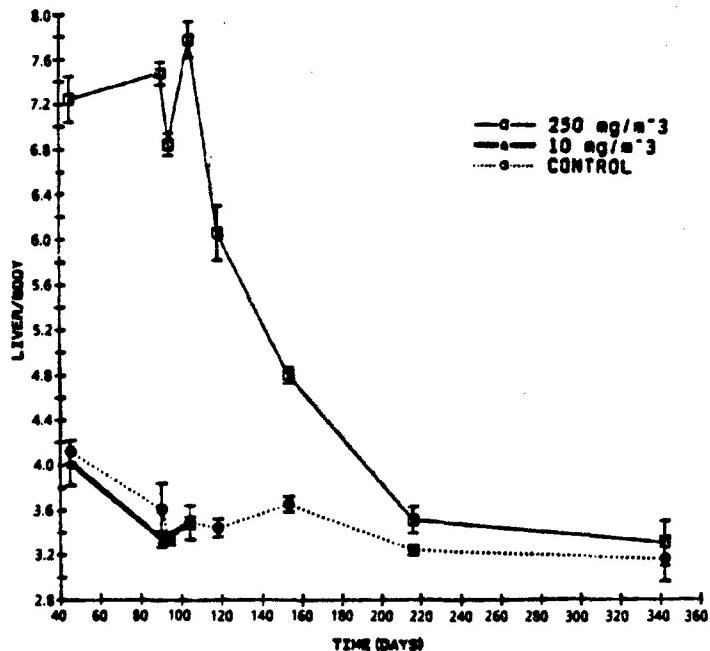
<sup>c</sup> Significantly different from control, p < 0.01, as determined by a one-factorial analysis of variance with multivariate comparisons.

<sup>d</sup> Fasted weights.

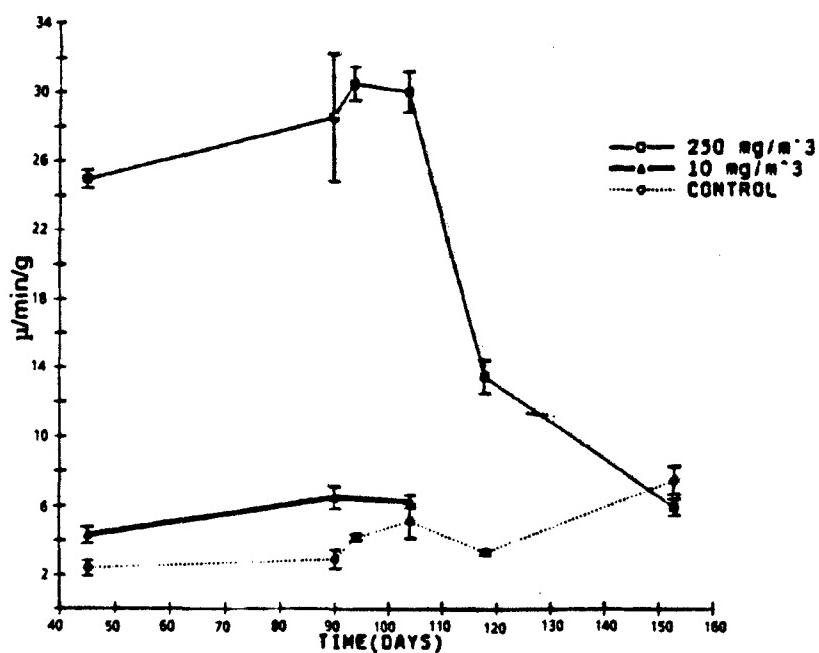
Significant increases in organ and relative organ weights were apparent in rats used for pharmacokinetic modeling at the 45-day interim sacrifice. Relative kidney weights of the 250-mg CTFE/m<sup>3</sup> group were greater than (p < 0.01) the low-concentration or control group. Relative liver weights were increased by >60% when compared to the low-concentration and control groups. At the conclusion of the 90-day study only liver weights were recorded. The relative liver weight at the scheduled exposure and postexposure serial sacrifice times are shown in Figure 3.3-3. Relative liver weights of the rats exposed to 10 mg CTFE/m<sup>3</sup> were not significantly different from controls at any of the sampling points.

Although the relative liver weights of the 10-mg CTFE/m<sup>3</sup>-exposed rats were not significantly different from controls, the rate of peroxisomal β-oxidation was 1.8 and 2.2 times that of the control group at 45 and 90 days, respectively (Figure 3.3-4). Two weeks following termination of the exposures the rate was not different from controls. The rate of peroxisomal β-oxidation measured in rats exposed at 250 mg CTFE/m<sup>3</sup> was 10.4 and 9.8 times that of controls at 45 and 90 days, respectively. Following termination of exposures, the rates decreased steadily, reaching that of control rats by 63 days postexposure.

This study is continuing and further data will be presented in future annual reports of THRU activities.



**Figure 3.3-3.** Liver/Body Weight Ratio of Male Rats following Inhalation Exposure to CTFE. Data were measured following 45 and 90 days exposure and at 4, 14, 28, 63, 126, and 252 days postexposure.



**Figure 3.3-4.** Average Rate of Peroxisomal  $\beta$ -Oxidation ( $\mu\text{m}/\text{min}/\text{g}$ ) in Livers of Male Rats following Inhalation Exposure to CTFE. Data were measured following 45 and 90 days exposure and at 4, 14, 28, and 63 days postexposure.

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### **3.4 RESOLUTION OF HEPATIC LESIONS IN RATS EXPOSED TO CHLOROTRIFLUOROETHYLENE OLIGOMERS**

H.G. Wall, E.R. Kinkead, and S.K. Bunger

#### **ABSTRACT**

The resolution of lesions that occur in the livers of rats that have been exposed subchronically to chlorotrifluoroethylene (CTFE) oligomer vapor plus aerosol was studied via light microscopic examination of the livers of groups of male Fischer 344 (F-344) rats that had been exposed to 0, 10, or 250 mg CTFE/m<sup>3</sup> for 6 h/day, 5 days/week for 13 weeks. Subgroups of rats from the 0- and 250-mg CTFE/m<sup>3</sup> concentration groups were necropsied immediately postexposure and on Days 2, 4, 7, 14, 28, 63, and 126 postexposure, and their livers were examined via light microscopy. Subgroups of rats from the 10-mg CTFE/m<sup>3</sup> group were necropsied immediately postexposure and on Days 2 and 14 postexposure only, and the livers from these rats also were studied via light microscopy.

#### **INTRODUCTION**

CTFE oligomer, a candidate hydraulic fluid, has been found to have low acute animal toxicity (Kinkead et al., 1987). An initial 90-day subchronic inhalation study disclosed that exposure levels 0.25, 0.50, and 1.00 mg CTFE/L produced dose- and sex-related toxic effects in male and female F-344 rats with the more pronounced toxic effects occurring in male rats (Kinkead et al., 1989). These exposure levels did not cause mortality among the experimentally exposed rats. A transient depression in body weight gain occurred in CTFE-exposed female rats during the first three weeks of exposure; whereas, the median and high concentrations caused significant reductions in the body weight gain of male rats for several weeks during the exposure period. The pathologic examinations disclosed dose- and sex-related hepatomegaly and increased liver-to-body weight ratios. The most marked hepatic effects occurred in male rats. The enlarged livers were characterized histologically by multifocal to diffuse hepatocytomegaly due to increased cytoplasm. The abundant granular eosinophilic cytoplasm seen in hematoxylin and eosin (H&E)-stained sections was demonstrated to have abundant peroxisomes and smooth endoplasmic reticulum when examined via transmission electron microscopy.

Preliminary data on the resolution of the liver lesions were obtained by the study of hepatectomy specimens obtained from four unexposed control male rats and two male rats that were exposed to 0.50 mg CTFE/L at 105 and 236 days postexposure, and by the study of liver specimens that were obtained when the animals were necropsied at one year postexposure (Kinkead et al., 1989). More severe hepatocytic degeneration was disclosed at 105 days postexposure as compared to immediate postexposure effects. Notably, more markedly enlarged hepatocytes with pronounced

cytoplasmic vacuolar degeneration or ground-glass cytoplasm were present and more necrotic cells were evident at 105 days postexposure. However, at 236 days and one year postexposure, hepatocytes of CTFE-exposed animals more closely resembled those of the control animals in that the cytoplasmic volume of the hepatocytes was less than at earlier time points. In addition, there was reduced cytoplasmic eosinophilic granularity and a return of the cytoplasmic basophilia associated with the normal staining pattern of hepatocytes due to the presence of RNA-rich organelles. Although this preliminary data suggested that the changes might represent an extended adaptive response by the liver, more extensive studies of the resolution of liver lesions were warranted.

Studies of phthalate esters and hypolipidemic agents have resulted in the association of hepatocytic peroxisome proliferation with the occurrence of an increased incidence of liver neoplasia in laboratory rodents (Reddy and Lalwani, 1983). Additionally, some chlorinated hydrocarbons have been shown to cause hepatocytic peroxisome proliferation (Elcombe et al., 1985; Goldsworthy and Popp, 1987; Odum et al., 1988). As part of a multidisciplinary research effort, the present morphologic study was initiated to obtain a more extensive characterization of the postexposure evolution of the morphologic effects in the livers of male rats that have been exposed subchronically to aerosolized CTFE oligomer.

This report describes the morphologic changes observed in the livers of rats from immediately after a 90-day exposure period to CTFE oligomer to 126 days postexposure.

#### MATERIALS AND METHODS

The design for a subchronic inhalation study to determine the no-effect level of CTFE oligomer included the allocation of study animals for the refinement of a physiologically based pharmacokinetic (PB-PK) model to describe the postexposure disposition of CTFE oligomer and for study of morphologic effects postexposure. Groups of four unexposed (control) rats and four rats each exposed to 0, 10, or 250 mg CTFE oligomer/m<sup>3</sup>, 6 h/day, five days per week for 13 weeks were necropsied at 0, 2, 4, 7, 14, 28, 63, and 126 days postexposure. The study is ongoing. Two groups of animals remain for sacrifice at 252 and 365 days postexposure, respectively. The experimental design (Section 3.3, Subchronic Inhalation Toxicity Studies on 3.1 Oil at Concentrations of 250, 50, and 10 mg/m<sup>3</sup>) and exposure environment (Section 3.7, Generation and Analysis of Monophasic and Biphasic Chlorotrifluoroethylene Oligomer Atmospheres: A Review) have been detailed elsewhere in the 1989 annual report.

In-life data acquisition included initial, weekly, and terminal body weights and at least twice daily observations for signs of illness. In-life data are included in Section 3.3 of the 1989 annual report.

At the designated necropsy interval, animals were weighed and then euthanized with carbon dioxide gas. Blood and tissue samples were collected rapidly for biochemical assessments required for the PB-PK model refinement. The weight of the liver was determined and recorded. Gross findings were recorded during prosection and tissues were collected and fixed in 10% neutral-buffered formalin for histopathology. Tissues for histopathology were embedded in paraffin, sectioned at four to seven microns, and stained with H&E (Luna, 1968). A one-way analysis of variance with Bonferroni multiple comparisons was used to analyze the mean severity data (Barcikowski, 1983).

## RESULTS

Results of the in-life phase of this study, including body and liver weight data are included in Section 3.3 of the 1989 Annual Report. This report focuses on the histopathologic alterations in the livers of rats at intervals up to 126 days after the termination of CTFE oligomer inhalation exposure.

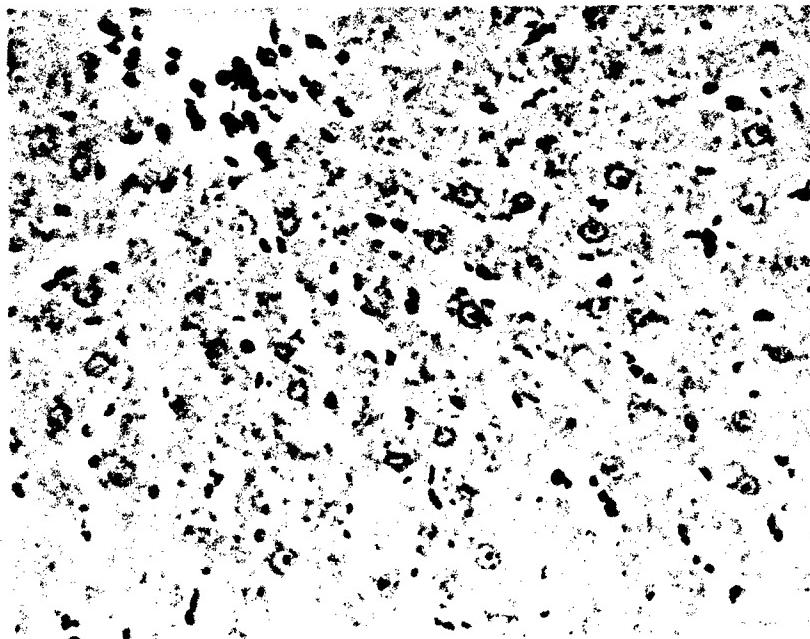
A detailed summary of the type and incidence of histologic lesions observed in the liver has been presented in Table 3.4-1. Representative photomicrographs of liver sections from control and CTFE-exposed rats are depicted in Figures 3.4-1 through 3.4-9. The livers of CTFE-exposed rats frequently had diffuse hepatocytomegaly with abundant eosinophilic granular cytoplasm. Subjectively, the degree of hepatocytomegaly was judged to be most severe in rats that received the 250-mg CTFE/m<sup>3</sup> exposure, and this group had a 100% incidence of hepatocytomegaly at each necropsy interval. The qualitative histopathological examinations of liver sections from rats exposed to 10 mg CTFE/m<sup>3</sup> disclosed no antemortem changes; however, slight postmortem hepatocytic swelling was evident in a few of the liver sections from rats necropsied on Days 0, 2, and 14 postexposure. At 63 days postexposure, the peripheral portions of the hepatic lobules contained isolated individual hepatocytes or aggregates of a few hepatocytes with markedly enlarged cytoplasmic compartments that contained numerous large, smoothly contoured cytoplasmic vacuoles. At 126 days postexposure, the foci of vacuolated cells were markedly larger and often were contiguous with altered areas in the periphery of adjacent lobules. Occasionally, necrotic cells were among the cells with vacuolar degeneration. The midzonal and centrolobular hepatocytes maintained their hepatocytomegaly and increased cytoplasmic granularity at 63 and 126 days postexposure, but did not have vacuolar degenerative changes or evidence of necrosis. Necrotic single cells were distributed sparsely in the liver sections of animals in the high-concentration exposure group at all necropsy intervals.

TABLE 3.4-1. LIVER HISTOLOGIC LESIONS INCIDENCE SUMMARY

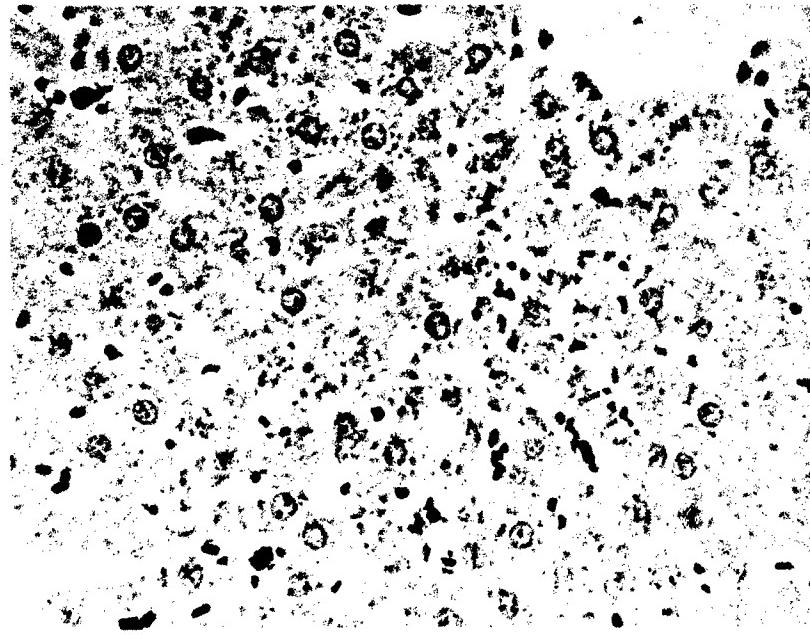
Liver Lesion <sup>b</sup>	Day 0			Day 2			Day 4			Day 7			Day 14			Day 28			Day 63			Day 126		
	0 <sup>a</sup>	10	250	0	10	250	0	250	0	10	250	0	250	0	250	0	250	0	250	0	250	0	250	
Number of animals examined	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	
Hepatocytomegaly	0	0	4	1	1	4	0	4	1	4	0	2	4	1	4	0	4	0	4	0	4	0	4	
Eosinophilic granular cytoplasm	0	0	4	1	0	4	1	4	1	4	0	0	4	0	4	0	4	0	4	0	4	0	4	
Reduced hepatocytic cytoplasmic basophilia	0	0	4	0	0	4	0	4	0	4	0	0	4	0	4	0	4	0	3	0	3	0	3	
Hepatocytic necrosis	0	0	4	0	0	4	0	4	0	4	0	0	1	0	4	0	4	0	4	0	1	0	1	
Hepatocytic vacuolar degeneration	0	0	0	0	0	0	0	1	0	0	0	1	3	0	3	0	3	0	3	0	0	0	0	
Inflammation	0	0	2	1	1	3	1	3	1	1	0	1	1	0	3	2	3	1	3	0	3	1	3	

• 0, 10, and 250 are the concentrations of CTFE oligomer expressed as milligram per cubic meter. Subgroups of animals exposed to 10 mg CTFE Oligomer/m<sup>3</sup> were necropsied only on Day 0 and Postexposure Days 2 and 14.

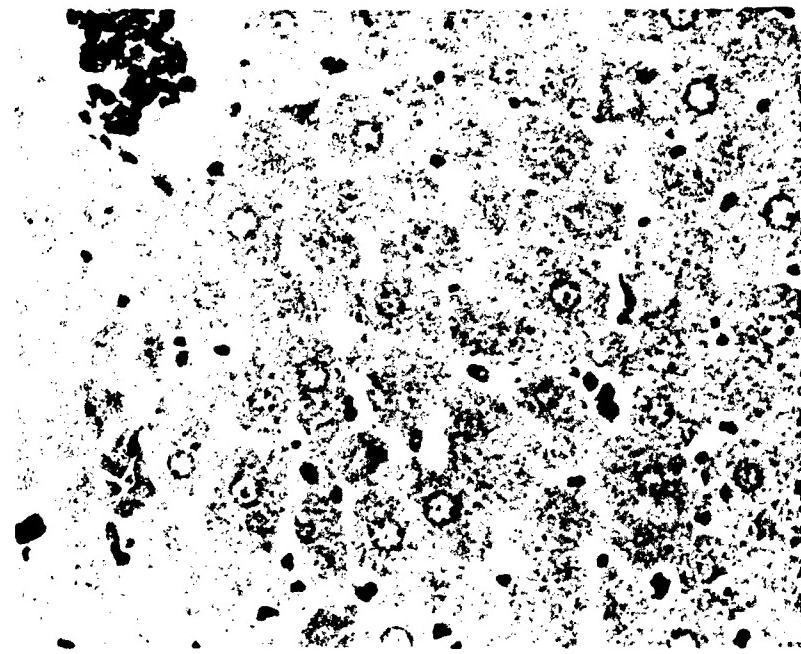
► The row of data adjacent to each lesion diagnosis contains the number of animals affected



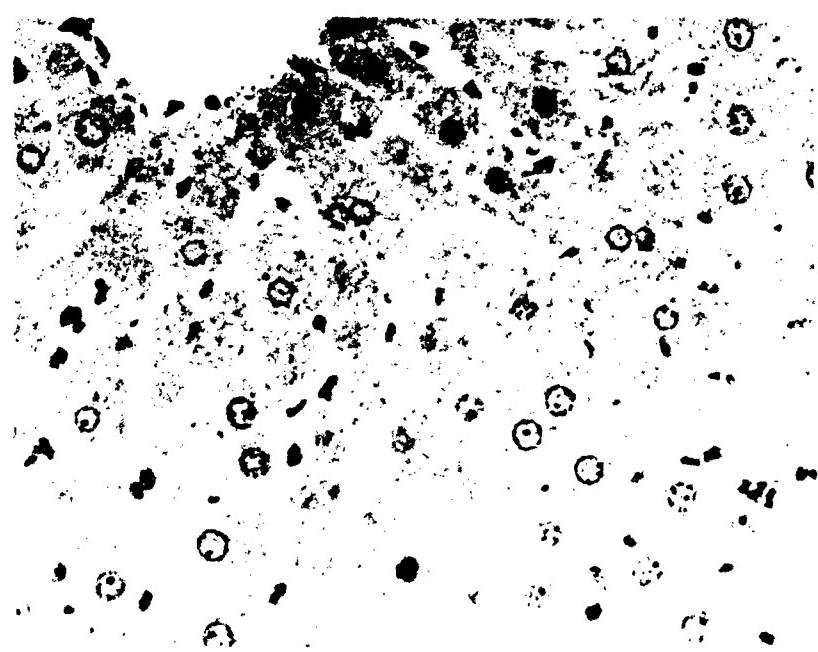
**Figure 3.4-1.** Photomicrograph of a Liver Section from a Control-Group Rat at the End of the Subchronic Exposure Period (Postexposure Day 0).



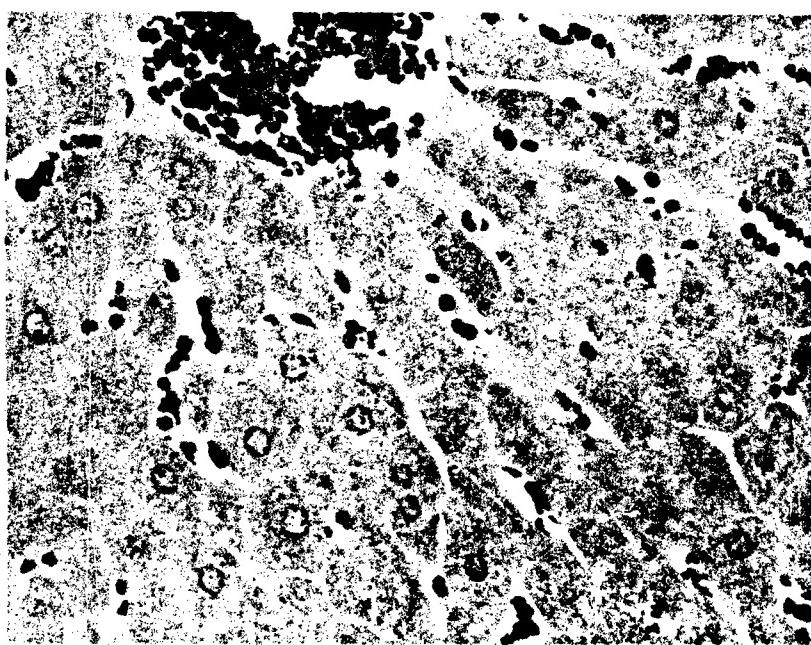
**Figure 3.4-2.** Photomicrograph of a Liver Section from a 10-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 0.



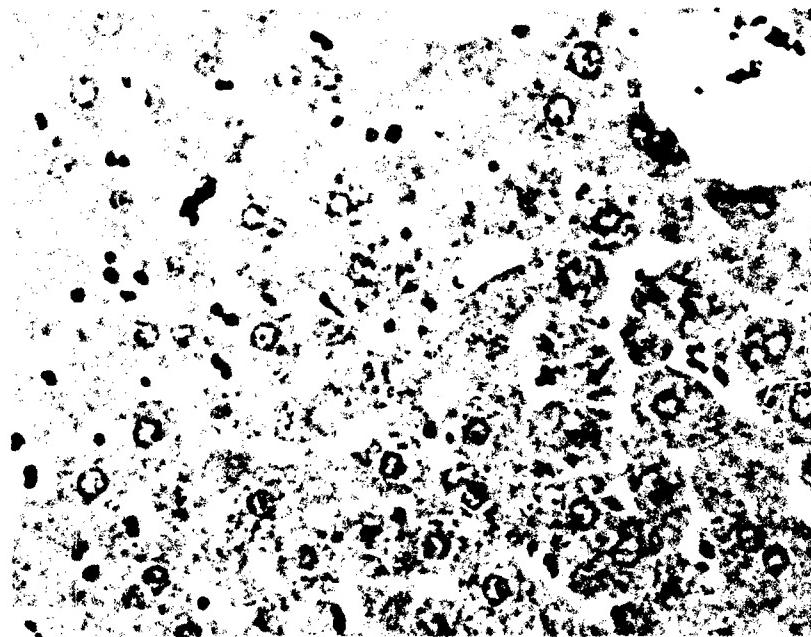
**Figure 3.4-3.** Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat Demonstrating Enlarged Hepatocytes with Granular Cytoplasm on Postexposure Day 0.



**Figure 3.4-4.** Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 7.



**Figure 3.4-5. Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 14.**



**Figure 3.4-6. Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 28.**

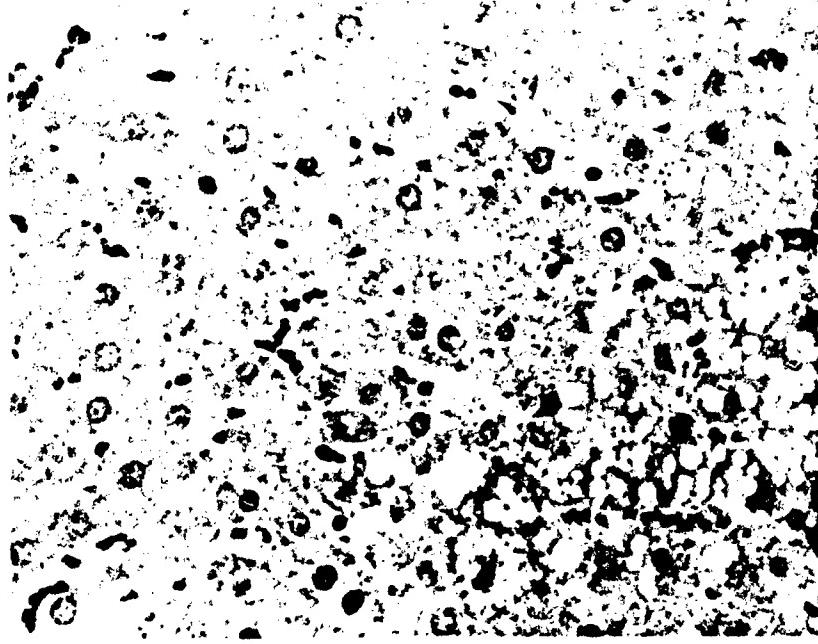


Figure 3.4-7. Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 63.

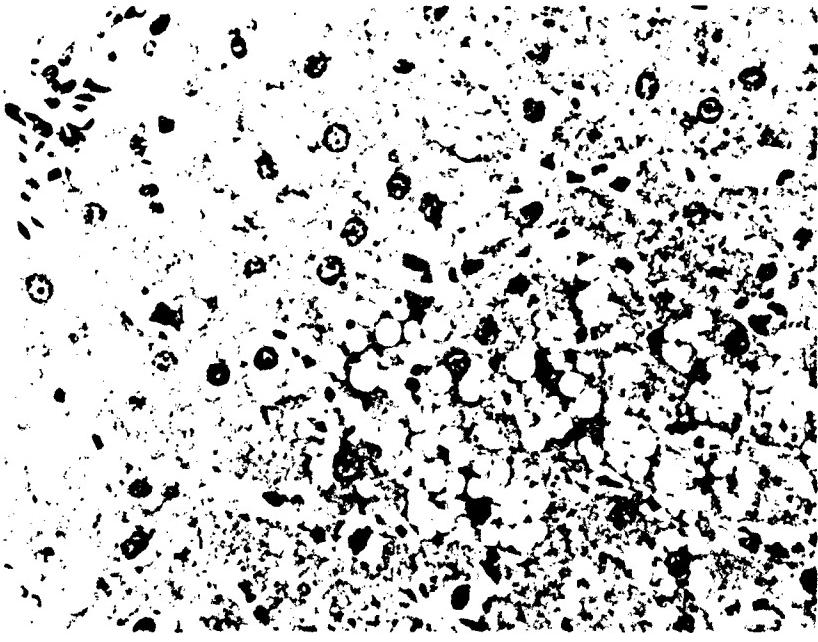


Figure 3.4-8. Photomicrograph of a Liver Section from a 250-mg CTFE/m<sup>3</sup> Group Rat on Postexposure Day 126.

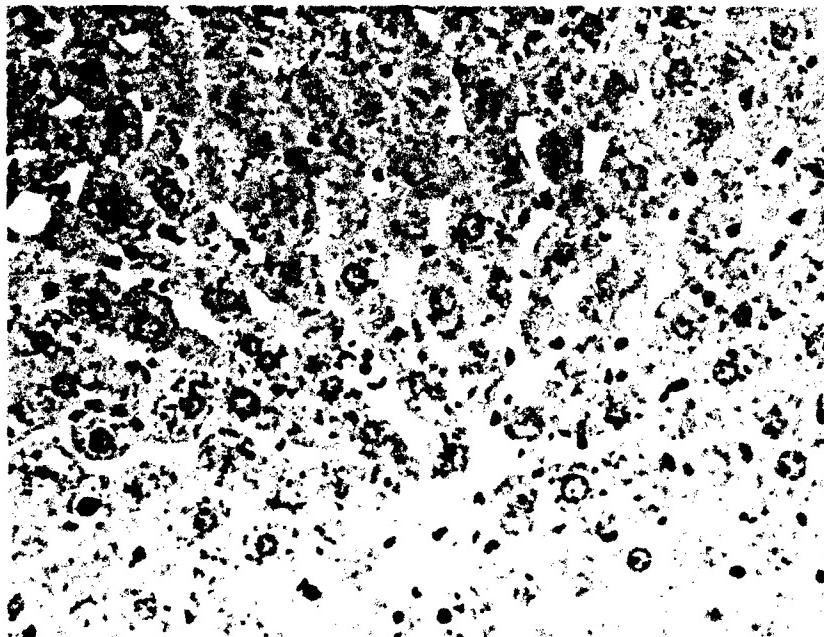


Figure 3.4-9. Photomicrograph of a Liver Section from a Control-Group Rat on Postexposure Day 126.

#### DISCUSSION

Although this study is incomplete, sufficient data was available to provide an interim analysis of the changes in rat livers following the cessation of a subchronic inhalation exposure. The antemortem histologic changes associated with exposure to CTFE oligomer were hepatocytomegaly, increased eosinophilic granularity, reduced cytoplasmic basophilia, hepatocytic necrosis, and hepatocytic cytoplasmic vacuolation (Table 3.4-2). The most severe hepatic alterations were observed most frequently in rats exposed to 250 mg CTFE/m<sup>3</sup>. The sequential liver histopathology indicated that hepatocytomegaly, hepatocytic eosinophilic granularity, and reduced hepatocytic basophilia in livers of the rats in the 250-mg CTFE/m<sup>3</sup> group were severe throughout the first 28 days after cessation of the exposure. The persistence of the severe effects may be due to continued dosing of the liver by CTFE oligomers released from depot sites within the animals. This possibility was initially suggested by Vinegar et al. (1989), when pharmacokinetic studies of inhaled CTFE oligomer in rats revealed that parent compound accumulated in fat could influence levels of the compound in blood and other tissues. Accumulated CTFE acid metabolites in the liver also have been postulated as the basis for hepatic changes (M.E. Andersen, personal communication). Further work involving identification and quantification of CTFE oligomer and its metabolites in animal tissues should help to confirm or refute the role of tissue depots of CTFE oligomer or CTFE oligomer metabolites in the pathogenesis of postexposure hepatic lesions. The hepatocytomegaly observed in control rats at Postexposure Days 2, 7, and 28, and in rats exposed to 10 mg CTFE/m<sup>3</sup> at Postexposure Days 2 and 14 was due to slight autolysis, as indicated by increased cytoplasmic volume and clear organelle-free space in the cytoplasmic compartment, particularly adjacent to the nucleus.

**TABLE 3.4-2. HISTOLOGIC LIVER LESIONS WITH SIGNIFICANT GROUP DIFFERENCES IN AVERAGE SEVERITY SCORES<sup>a</sup>**

Day Postexposure	Hepatocyteomegaly			Eosinophilic Cytoplasmic Granularity			Reduced Cytoplasmic Basophilia <sup>b</sup>		
	0b,f	10b	250b	0b,f	10b	250b	0b,f	10b	250b
0	0	0	4.25 ± 0.50c,d	0	0	4.0 ± 0c,d	0	0	4.0 ± 0c,d
2e	0.25 ± 0.50	0.33 ± 0.58	4.75 ± 0.50c,d	0.25 ± 0.50	0	4.00 ± 0c,d	0	0	4.0 ± 0c,d
4	0	NA	4.75 ± 0.50c	0.25 ± 0.50	NA	5.0 ± 0c	0	NA	4.75 ± 0.5c
7	0.25 ± 0.50	NA	5.0 ± 0c	0.25 ± 0.50	NA	5.0 ± 0c	0.25 ± 0.50	NA	5.0 ± 0c
14	0	0.50 ± 0.58	4.75 ± 0.50c,d	0	0	5.0 ± 0c,d	0	0	5.0 ± 0c,d
28	0.50 ± 1.30	NA	4.25 ± 0.50c	0	NA	4.50 ± 0.58c	0	NA	4.50 ± 0.58c
63	0	NA	2.50 ± 0.58c	0	NA	2.25 ± 0.50c	0	NA	2.25 ± 0.50c
126	0	NA	2.0 ± 0c	0	NA	0	0	NA	0
Day Postexposure	Hepatocytic Necrosis			Cytoplasmic Vacuolation					
	0b,f	10b	250b	0b,f	10b	250b			
0	0	0	1.75 ± 0.50c,d	0	0	0			
2	0	0	1.75 ± 0.50c,d	0	0	0			
4	0	NA	1.25 ± 0.50c	0	NA	0.5 ± 1.0			
7	0	0	0.50 ± 1.0c	0	NA	0			
14	0	0	0.50 ± 1.0	0	0.50 ± 1.0	1.5 ± 1.0			
28	0	NA	1.25 ± 0.50c	0	NA	1.5 ± 1.0			
63	0	NA	2.00 ± 0c	0	NA	3.0 ± 2.0c			
126e	0	NA	0.25 ± 0.50	0	NA	0			

<sup>a</sup> Average severity scores of lesions are listed as the mean ± SD; NA = not applicable (no animals from the exposure group were necropsied); and the number of rat livers examined per group was four unless indicated otherwise.

<sup>b</sup> 0, 10, and 250 are the concentrations of CTFE oligomer expressed as milligrams per cubic meter.

<sup>c</sup> Significantly different from the 0-mg CTFE/m<sup>3</sup> group at  $p < 0.01$ , using a one-way analysis of variance with Bonferroni multiple comparisons.

<sup>d</sup> Significantly different from the 10-mg CTFE/m<sup>3</sup> group at  $p < 0.01$ , using a one-way analysis of variance with Bonferroni multiple comparisons.

<sup>e</sup> The number of rat livers examined was three for the 10-mg CTFE/m<sup>3</sup> group.

<sup>f</sup> The number of rat livers examined was three for the 0-mg CTFE/m<sup>3</sup> group.

Changes associated with resolution of the hepatocytic effects of CTFE oligomer exposure were not apparent in the high-concentration group until 63 days postexposure. At that time and at 126 days postexposure, there was less marked hepatocytomegaly and eosinophilic cytoplasmic granularity, and the cytoplasmic basophilia attributable to RNA-rich organelles was more prominent. Although these changes were suggestive of resolution, foci of ballooned hepatocytes and foci of hepatocytic necrosis were prominent degenerative changes. At 126 days postexposure, hepatocytic necrosis and hepatocytic vacuolar degeneration in the livers of rats from the high-concentration group was more severe than at 63 days postexposure. Hepatocytic necrosis may be part of the resolution process. Observations at 252 days postexposure and later may assist in the interpretation of the significance of the hepatocytic necrosis. The preliminary data from a few animals that were examined at 105, 236, and 365 days after cessation of a 13-week CTFE oligomer inhalation exposure at 0.5 mg/L demonstrated peripherolobular hepatocytic degeneration and foci of necrosis at 105 days postexposure and reduced cytoplasmic volume, reduced eosinophilic cytoplasmic granularity, and more abundant cytoplasmic basophilia at 236 and 365 days postexposure (Kinkead et al., 1989). Additionally, at 236 and 365 days postexposure, there was biliary duplication in the livers of CTFE-exposed and control rats. However, hepatic fibrosis was not observed.

The primary toxicologically significant effect of CTFE oligomer exposure is peroxisome proliferation, an effect that has been associated with the induction of liver tumors in rats and mice administered other chemicals that cause peroxisome proliferation (Reddy and Lalwani, 1983). An initiation/promotion study of the model carcinogenic peroxisome proliferator, Wy-14,643, administered as a liver tumor promoter to F-344 rats for 52 weeks disclosed that the compound caused a rapid increase in the size of basophilic (hyperplastic) liver foci without increasing the number of foci (Cattley and Popp, 1989). Through 126 days postexposure, preneoplastic foci were not detected in either control or CTFE-exposed rats.

These interim study findings have provided some indication that rat liver lesions resolve after cessation of CTFE oligomer exposure, but not before a postexposure increase in the extent and severity of hepatocytic vacuolar degeneration and necrosis (Postexposure Days 63 and 126).

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### **3.5 REPEATED-DOSE GAVAGE STUDIES ON CHLOROTRIFLUOROETHYLENE USING LEGHORN HENS**

**E.R. Kinkead, S.K. Bunger, R.E. Wolfe, C.S. Godin, and H.G. Wall**

#### **ABSTRACT**

Halocarbon 3.1 oil has been shown to cause liver hypertrophy and peroxisomal proliferation in Fischer 344 (F-344) rats. Hens received repeated oral doses of 1.25 g chlorotrifluoroethylene (CTFE)/kg over a two-week period. No effects were noted in liver to body weight ratios or in peroxisomal  $\beta$ -oxidation levels at sacrifice. Histopathologic examination of livers revealed no treatment-related lesions in any of the test animals.

#### **INTRODUCTION**

Long-term inhalation exposure of F-344 rats to oligomers of CTFE blended to a viscosity of 3.1 (halocarbon 3.1 oil) has resulted in increases in liver to body weight ratios and peroxisomal  $\beta$ -oxidation levels as well as peroxisomal proliferation within hepatocytes (Kinkead et al., 1989a). Repeat oral dosing of F-344 rats with a similar CTFE oligomer resulted in increased liver to body weight ratios (Kinkead et al., 1989b); however, transmission electron microscopy was not performed and it is not certain that the compound caused peroxisomal proliferation. The livers of chickens have been shown to respond markedly to peroxisome proliferators when compared to other nonrodent species (Reddy et al., 1984). It is believed that CTFE is metabolized through the  $\Omega$ -oxidation pathway resulting in peroxisomal proliferation in the rat. In order to confirm the involvement of the  $\Omega$ -oxidation pathway and examine the effects of CTFE on livers of a species other than rats, chickens were gavaged repeatedly with CTFE.

#### **MATERIALS AND METHODS**

##### **Animals**

Debeaked female leghorn hens (*Gallus domestica*, Carey Nick 320 hybrid, Carey Farms, Inc., Marion, OH), four to six months of age and weighing between 1.4 and 1.7 kg, were used in this study. The hens were identified by leg bands and were group housed in 3 ft  $\times$  6 ft pens to allow free movement. Food (MannaPro, Eggmaker 15 Crumbles) and water were provided ad libitum. Hens were maintained on a 12-h light/dark cycle, beginning at 0300 h.

The supplier provided additional flock history and information on husbandry practices. Table 3.5-1 lists the vaccinations administered to the flock. No pesticides were applied to hens used in this study, nor were disinfectants used while birds were in the poultry houses. When poultry houses became vacant, they were cleaned and disinfected with formaldehyde. The supplier indicated that the flock had not experienced any disease problems.

TABLE 3.5-1. FLOCK VACCINATION HISTORY\*

Vaccination	Age of hen
Marek's disease	1 day
Infectious bronchitis	2 weeks
Infectious bursal disease	2 weeks
Newcastle disease	2 weeks
Infectious bronchitis (booster)	10-12 weeks
Newcastle disease (booster)	10-12 week
Fowlpox	20-24 weeks

\*Provided verbally by Carey Farms, Inc., Marion, OH.

#### **Test Materials**

The CTFE sample was supplied by the U.S. Air Force and identified as shown below.

CTFE 3.1 Oil  
MLO 87-124  
Safetol® 3.1  
Batch # 86-134

This CTFE oligomer mixture contained 0.1% (vol/vol) of a rust inhibitor additive, neutral barium dinonylnaphthalene sulfonate. A second additive was 0.05% (vol/vol) of a proprietary compound (composition unknown).

Chemically, halocarbon oils like CTFE are saturated, low molecular weight polymers of CTFE having the general formula  $(CF_2CFCI)_n$ . They are made using a controlled polymerization technique and are stable – the terminal groups are completely halogenated. The products then are separated by vacuum distillation into various fractions, from light oils to waxes.

#### **Dosing Regimen**

CTFE was administered to a group of 10 unfasted hens during a two-week period (10 doses over 14 days). The fluid was administered neat at 1.25 g CTFE/kg by oral intubation employing a 3-cc syringe fitted with a 15-cm infant feeding catheter. Each hen was weighed weekly for calculation of daily dose volumes. An additional group of five hens was dosed on the same schedule with distilled water and served as controls.

All hens were euthanized at the conclusion of the 14-day period, at which time the livers were removed and weighed. Sections of liver were sampled for peroxisomal  $\beta$ -oxidation assays and for light and electron microscopy.

### **Enzyme Assay**

Peroxisomal  $\beta$ -oxidation assays were performed on approximately 1 g of liver tissue. Samples were removed from the median lobe and placed in ice-cold 0.25 M sucrose. The sample was homogenized, then centrifuged at 1500  $\times g$  for 10 min. A portion of the resultant supernatant was used to measure the rate of palmitoyl coenzyme A (CoA) oxidation.

### **Statistics**

A two-factorial analysis of variance was used for the enzyme assay. Histopathologic results will be evaluated using a Fisher's Exact Test or Yates Corrected Chi-Square (Zar, 1974).

### **EXPERIMENTAL RESULTS**

No deaths resulted from the oral administration of the test agent and no signs of toxicity were observed during the 14-day test period. One control hen died of extraneous causes following the seventh treatment. Mean body weights of the hens are listed in Table 3.5-2.

**TABLE 3.5-2. MEAN<sup>a</sup> BODY WEIGHTS OF CHICKENS ORALLY DOSED WITH CTFE**

	Control <sup>b</sup>	CTFE <sup>c</sup>
Day 2	1.53 $\pm$ 0.05	1.51 $\pm$ 0.02
Day 5	1.44 $\pm$ 0.02	1.42 $\pm$ 0.02
Day 12	1.30 $\pm$ 0.15 <sup>d</sup>	1.35 $\pm$ 0.05
Day 14	1.53 $\pm$ 0.06 <sup>d</sup>	1.39 $\pm$ 0.03

<sup>a</sup> Mean  $\pm$  SEM.

<sup>b</sup> N = 5.

<sup>c</sup> N = 10.

<sup>d</sup> N = 4.

Mean liver weights and liver enzyme values are listed in Table 3.5-3. Relative liver weights of hens dosed with CTFE were not different from those treated with distilled water. Likewise, peroxisomal  $\beta$ -oxidation levels of the test hens were similar to control levels.

**TABLE 3.5-3. LIVER EFFECTS EXAMINED AT NECROPSY**

Treatment Group	Liver Weight	Liver to Body Weight Ratio	Peroxisomal $\beta$ -Oxidation <sup>a</sup>
1.25 g CTFE/kg <sup>b</sup>	36.3 $\pm$ 2.5	2.6 $\pm$ 0.2	0.4 $\pm$ 0.1 <sup>c</sup>
Control <sup>d</sup>	41.0 $\pm$ 3.9	2.7 $\pm$ 0.2	0.5 $\pm$ 0.3

<sup>a</sup> Micromoles per minute per gram of liver.

<sup>b</sup> N = 10.

<sup>c</sup> N = 7.

<sup>d</sup> N = 4.

Gross pathological evaluations performed at sacrifice determined two hens with lesions. One hen had dark purple areas on the ventral surface of the stomach; the second had a pale liver. Both

hens were controls and had been treated with distilled water only. No gross lesions were observed in the hens treated with CTFE. Histopathologic examination determined that no hens treated with CTFE had lesions that were considered related to chemical exposure.

#### **DISCUSSION**

Previous experience in this laboratory has shown that animals that respond to chemical insults by the production of hepatic peroxisome proliferation also demonstrate increases in relative liver weights and in peroxisomal  $\beta$ -oxidation levels (Kinkead et al., 1989a, 1989b). Even though hens have been shown to respond to peroxisome proliferators (Reddy et al., 1984), the hens in this study did not have the expected increases in either relative liver weights or in enzyme levels. It appears that CTFE is metabolized in hens in a manner that does not result in the formation of long-chain or medium-chain fatty acids. The reason for the lack of increased fatty acyl-CoA oxidation or liver size is unknown and perhaps can be answered by further investigation of the CTFE uptake in hens.

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### **3.6 ASSESSMENT OF THE POTENTIAL GENOTOXICITY OF CHLOROTRIFLUOROETHYLENE OLIGOMER**

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#### **ABSTRACT**

Chlorotrifluoroethylene (CTFE) oligomer has been proposed as a hydraulic fluid for the high-pressure systems of the Advanced Tactical Fighter. This material, predominantly C6 and C8 fully halogenated molecules, was evaluated in *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the Ames *Salmonella*/microsomal mutagenicity assay, the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus Chinese hamster ovary (CHO) gene mutation assay, the CHO/sister chromatid exchange (SCE), and chromosome aberration assay. All were conducted both in the absence and the presence of S9 fraction microsomal enzymes. CTFE did not produce positive findings in any of the assays with the exception of the HGPRT mammalian forward mutation assay where it was evaluated as weakly positive.

#### **INTRODUCTION**

Chlorotrifluoroethylene oligomer, a perhalogenated polymeric mixture of CTFE trimers and tetramers end capped with chlorine, has potential as a hydraulic fluid in advanced Department of Defense weapons systems. The material did not demonstrate acute toxicity with the exception of slight eye irritation. However, sensitization studies indicated that it may produce an allergic response with prolonged skin contact (Kinkead and Culpepper, 1989). Subchronic inhalation exposures to 0.25, 0.50, and 1.00 mg CTFE/L all resulted in hepatocytomegaly in both sexes of Fischer 344 rats (Kinkead et al., 1989). Electron microscopic examination of livers from the exposed animals demonstrated peroxisomal proliferation and increased amounts of smooth endoplasmic reticulum as the primary structural factors responsible for the hepatocytomegaly. To evaluate more fully the potential human health hazards of this hydraulic fluid, *in vitro* tests were conducted to determine its capacity to cause genotoxicity.

#### **MATERIALS AND METHODS**

The *Salmonella* reverse mutation assay (Ames et al., 1975), using a preincubation method (Yahagi et al., 1975), was employed to examine mutagenic activity. This assay evaluated the test article and/or its metabolites for ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes. The tester strains used

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in this study were TA98, TA100, TA1535, TA1537, and TA1538. Following toxicity testing, six concentrations of CTFE were tested, from 333 to 10,000 µg/plate, both in the absence and presence of S9 metabolizing enzymes. By convention the doses have been expressed as micrograms of CTFE per plate to reflect the fact that exposure of the test system to the test article did not cease at the end of the 20-min preincubation period.

To evaluate the ability of CTFE to induce forward mutations in mammalian cells, the HGPRT locus in CHO cells was used in an *in vitro* assay both with and without metabolic activation. Based on preliminary range-finding cytotoxicity assays, treatment conditions chosen for the nonactivation mutation assay ranged from 0.05 to 5.0 mg/mL, and the treatment conditions for the S9-activation portion of the mutation assay ranged from 0.1 to 5.0 mg/mL. The assay procedures were based on those reported by Hsie et al. (1975) and reviewed by Hsie et al. (1981), with modifications suggested by Myhr and DiPaolo (1978).

In the SCE assay, CHO cell cultures that were exposed to CTFE for approximately two cell cycles were analyzed to determine cellular toxicity and the effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to allow for the progression to second generation cells. The doses used in the assay ranged from 0.005 to 0.5 mg CTFE/mL without metabolic enzymes and from 0.05 to 5.0 mg CTFE/mL in the presence of metabolizing enzymes.

Cell cycle kinetics from the SCE assay were used to (1) determine the dose range to be used in the chromosomal aberrations assay and (2) determine the optimal time of harvest of the dosed cells so that primarily metaphase cells, in the first metaphase since exposure, would be analyzed for chromosomal aberrations. For CTFE-treated cultures a 10-h harvest was conducted for both the nonactivation and the activation assay. Chromosomal aberrations were analyzed from the four highest doses from which results could be obtained.

## RESULTS

### *Ames Salmonella Reverse Mutation Assay*

The use of CTFE in *Salmonella* reverse mutation studies did not result in increased mean numbers of revertants per plate with any of the tester strains, either in the presence or absence of S9.

### *HGPRT Mammalian Forward Mutation Assay*

In preliminary cytotoxicity assays, under nonactivation test conditions using CHO-K1-BH<sub>4</sub> cultures, CTFE was nontoxic from 0.005 to 0.1 mg/mL, followed by increased toxicity from 0.2 to 2.0 mg/mL. Total cell killing was observed at 5.0 mg CTFE/mL. In the presence of activation enzymes, no toxicity was observed from 0.005 to 0.5 mg/mL, followed by increased toxicity from 1.0 mg/mL to

the maximum dose tested, 5.0 mg/mL. Treatment with 5.0 mg CTFE/mL resulted in a 14.2% relative clonal survival. Treatment conditions selected for the nonactivation portion of the mutation assay ranged from 0.05 to 5.0 mg CTFE/mL and for the metabolic activation portion of the mutation assay the treatment conditions ranged from 0.1 to 5.0 mg CTFE/mL.

Two independent mutation assays were performed with the test material using nonactivation conditions (Tables 3.6-1 and 3.6-2) and concentrations that demonstrated dose-related toxicity. In the first trial, concentrations ranged from 0.05 to 5.0 mg CTFE/mL (Table 3.6-1). The 5.0 mg/mL culture had less than 10% relative clonal survival and was terminated prior to plating for mutant selection. Two nontoxic concentrations, 0.05 and 0.4 mg/mL, were not plated for mutant selection due to a sufficient number of nontoxic concentrations available for analysis. In this trial the mutant frequencies of CTFE-treated cultures were within the acceptable range for background mutant frequency variation (0 to  $15 \times 10^{-6}$ ), with the exception of the cultures treated with 0.1 and 2.0 mg CTFE/mL. Those cultures had mutant frequencies of  $18.1 \times 10^{-6}$  and  $27.4 \times 10^{-6}$ , respectively, which were significantly greater than the mutant frequencies of the concurrent vehicle control cultures. The mutant frequencies of the other cultures varied randomly with dose and no other culture had a significantly elevated mutant frequency. Assay evaluation criteria required a second mutation assay to properly evaluate the elevated mutant frequency at the highest acceptable concentration. The second nonactivation mutation assay (Table 3.6-2) used a modified dose range to focus on the toxic range where significance was observed in the first trial. Six concentrations were used (0.1 to 5.0 mg/mL) with relative survivals that ranged from 87.5 to 3.8% (Table 3.6-2). The mutant frequencies of all the treated cultures varied randomly with dose and were within the range for acceptable background mutant frequencies with the exception of the culture treated with the highest concentration, 5.0 mg/mL. This culture had a mutant frequency of  $19.0 \times 10^{-6}$ , which was significantly greater than the mutant frequencies of the concurrent vehicle controls.

Under S9 metabolic activation test conditions, the cultures treated with CTFE (0.1 to 5.0 mg/mL) showed a dose-related decrease in both relative survival and relative population growth. An intermediate concentration, 1.5 mg/mL, was not plated for mutant selection due to a sufficient number of nontoxic concentrations available for analysis. Seven concentrations were plated and analyzed. With S9 metabolic activation, the mutant frequency of cultures treated with CTFE varied within the acceptable range of vehicle control mutant frequency variation. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was both significantly greater than the average background mutant frequency of the concurrent vehicle controls and higher than the acceptable range of background mutant frequencies. However, three random cultures, with mutant frequencies within the acceptable background variation range, had mutation frequencies significantly greater than the concurrent vehicle control.

**TABLE 3.6-1. EFFECT OF CHLOROTRIFLUOROETHYLENE OLIGOMER ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS: TRIAL 1**

Nonactivation <sup>a</sup> Test Condition	Relative Population Growth (% of Control)	Mutant Colonies Dish Number												Total Mutant Colonies	Absolute C.E. ± SD (%)	Mutant Frequency in 10 <sup>-6</sup> Units <sup>b</sup>
		1	2	3	4	5	6	7	8	9	10	11	12			
Vehicle Control <sup>c</sup>	109.3	4	0	0	1	0	1	0	3	0	2	4	6	21	96.2 ± 14.8	9.1
Vehicle Control <sup>c</sup>	90.5	1	1	0	2	0	0	0	1	2	1	1	3	12	97.7 ± 6.7	5.1
Positive Control (50 µg/mL BrdU) <sup>d</sup>	40.5	23	14	17	20	24	24	14	18	20	21	22	25	242	88.9 ± 5.1	113.4 <sup>e</sup>
Test Article (mg/mL)																
0.05	NS <sup>f</sup>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.1	70.0													42	96.9 ± 9.4	18.1 <sup>g</sup>
0.2	81.9													4	95.4 ± 1.3	1.7
0.3	87.6													9	104.2 ± 5.5	3.6
0.4	NS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.5	81.5													12	109.5 ± 4.3	.6
0.75	45.8													12	88.2 ± 3.1	5.7
1.0	47.0													12	99.5 ± 5.2	5.0
2.0	— <sup>h</sup>													62	94.4 ± 8.6	27.4 <sup>g</sup>
5.0	T <sup>i</sup>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

<sup>a</sup> Conducted in the absence of S9 fraction metabolizing enzymes.

<sup>b</sup> Mutant frequency = Total mutant colonies/(no. of dishes × 2 × 105 × absolute C.E.).

<sup>c</sup> Vehicle control = 1% Pluronic<sup>®</sup> F-68.

<sup>d</sup> BrdU = 5-Brorno-2'-deoxyuridine.

<sup>e</sup> Significant increase: Kastenbaum Bowman test p ≤ 0.01 and mutant frequency ≥ 15 × 10<sup>-6</sup>.

<sup>f</sup> NS = Not plated for selection due to sufficient number surviving higher dose levels.

<sup>g</sup> T = Terminated due to excessive toxicity.

<sup>h</sup> C.E. = Cloning efficiency.

#### Sister Chromatid Exchange Assays

In the SCE assay without metabolic activation, complete cellular toxicity was observed at 500, 1670, and 5000 µg/mL. Severe cellular toxicity was observed at 167 µg/mL with a dose-dependent reduction in toxicity seen at 50 and 16.7 µg/mL. Results were evaluated from cultures treated with 5, 16.7, 50, and 167 µg CTFE/mL. No significant increase in SCE was observed at the concentrations analyzed.

In the SCE assay with metabolic activation, severe toxicity was observed at 1670 µg/mL, and complete cellular toxicity was observed at 5000 µg CTFE/mL. No toxicity was discernible at the subsequent concentration of 500 µg/mL. Results were evaluated at 50, 167, 500, and 1670 µg/mL (Table 3.6-2). A weakly positive increase in SCE was observed only at 500 µg/mL and not at the other concentrations analyzed. This increase was not substantiated at the higher dose of 1670 µg/mL, and the increase at 500 µg/mL was not significantly greater than the results in the negative control culture.

**TABLE 3.6-2. EFFECT OF CHLOROTRIFLUOROETHYLENE OLIGOMER ON THE MUTATION FREQUENCY OF CHINESE HAMSTER OVARY CELLS: TRIAL 2**

Nonactivation <sup>a</sup> Test Condition	Relative Population Growth (% of Control)	Mutant Colonies Dish Number												Total Mutant Colonies	Absolute C.E. ± SD (%)	Mutant Frequency in 10 <sup>-6</sup> Units <sup>b</sup>
		1	2	3	4	5	6	7	8	9	10	11	12			
Vehicle Control <sup>c</sup>	108.6	2	1	2	0	1	0	0	0	1	0	1	1	9	80.5 ± 5.3	4.7
Vehicle Control <sup>c</sup>	91.4	1	2	0	2	1	2	1	1	1	3	2	4	20	78.0 ± 4.8	10.7
Positive Control (50 µg/mL BrdU) <sup>d</sup>	51.1	13	10	11	10	13	14	11	9	13	15	8	19	146	73.5 ± 1.3	82.8 <sup>e</sup>
Test Article (mg/mL)																
0.1	160.1	1	1	5	0	1	1	3	1	2	0	2	0	17	79.4 ± 1.6	8.9
1.0	48.3	1	2	0	0	0	0	1	1	0	1	0	0	6	85.7 ± 2.5	2.9
2.0	29.2	2	0	2	0	0	0	2	0	0	0	0	0	6	81.0 ± 4.8	3.1
3.0	18.7	0	0	0	0	0	0	0	0	0	1	1	2	2	92.2 ± 8.3	0.9
4.0	8.2	1	0	1	0	0	1	1	1	0	3	0	0	5	82.0 ± 10.5	2.5
5.0	4.1	1	1	4	5	3	4	3	4	3	1	6	1	36	78.9 ± 5.0	19.0 <sup>e</sup>

<sup>a</sup> Conducted in the absence of S9 fraction metabolizing enzymes.

<sup>b</sup> Mutant frequency = Total mutant colonies/(no. of dishes × 2 × 10<sup>5</sup> × absolute C.E.).

<sup>c</sup> Vehicle control = 1% Pluronic<sup>®</sup> F-68.

<sup>d</sup> BrdU = 5-Bromo-2'-deoxyuridine.

<sup>e</sup> Significant increase: Kastenbaum Bowman test p ≤ 0.01 and mutant frequency ≥ 15 × 10<sup>-6</sup>.

C.E. = Cloning efficiency.

### Chromosomal Aberrations Assays

Under nonactivation conditions, cell cycle kinetics from the SCE assay indicated only a slight cell cycle delay at 167 and 50 µg/mL. A 10-h harvest was selected for the aberrations assay testing a dose range of 20 to 300 µg CTFE/mL. Severe toxicity was observed at 300 µg/mL; however, no toxicity was discerned at the subsequent concentration of 250 µg/mL. Results were evaluated at 150, 200, 250, and 300 µg/mL and no significant increase in chromosomally aberrant cells was observed.

Cell cycle kinetics from the SCE assay with metabolic activation indicated only a slight cell cycle delay at 1670 µg CTFE/mL and a 10-h harvest was selected for the aberrations assay testing concentrations of 200 to 2500 µg/mL. Severe toxicity was observed at 2500 µg/mL. No toxicity was observed at the subsequent concentration of 2000 µg/mL. Results were evaluated at 1000, 1500, 2000, and 2500 µg/mL and no significant increase in chromosomally aberrant cells was observed.

### DISCUSSION

CTFE and its potential metabolites, resulting from S9 fraction activation, were found not to have genotoxic potential under the conditions of the *Salmonella* reverse mutation, the SCE, and chromosome aberration assays. Also, in the presence of metabolizing enzymes CTFE was assessed as negative in the HGPRT mammalian cell mutation.

Under the conditions of the HGPRT locus assay in CHO cells in the absence of metabolizing enzymes, CTFE was considered weakly positive for inducing forward mutations. Two independent trials that included toxic concentrations had cultures with significantly increased mutant frequencies. Although toxic concentrations did show a significant increase in mutant frequency in both trials, there was no agreement between the two trials as to the degree of toxicity required for a response. Assay evaluation criteria, established a priori, require that a dose-related or toxicity-related increase in mutant frequency be observed, or, if an increased mutant frequency is observed only at a single concentration the number of mutant colonies observed must be more than twice that needed for a statistically significant response and must exceed  $15 \times 10^6$  to compensate for random fluctuations in background frequencies that are typical to this assay. Neither trial alone had a sufficient response for a positive evaluation as defined by the assay evaluation criteria. With data from two trials available for review, CTFE was evaluated as weakly positive for inducing forward mutations at the HGPRT locus in CHO cells under the nonactivation conditions used in the study.

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### **3.7 GENERATION AND ANALYSES OF MONOPHASIC AND BIPHASIC CHLOROTRIFLUOROETHYLENE OLIGOMER EXPOSURE ATMOSPHERES: A REVIEW**

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#### **ABSTRACT**

Recently a series of experiments were conducted to determine the acute and subchronic inhalation toxicity of chlorotrifluoroethylene (CTFE) oligomer-based hydraulic fluids in which exposure concentrations varied by three orders of magnitude. Furthermore, these exposure atmospheres were physicochemically heterogeneous, in that the relative concentrations of the constituents of the oligomeric mixture and physical characteristics of the atmospheres (vapor vs. vapor and aerosol) varied simultaneously as a function of concentration. Hence, it was necessary to adopt atmosphere generation methods and analytical strategies that would address these physicochemical complexities consistently over a broad range of concentrations. Generation methods were developed by which the exposure atmospheres remained chemically comparable to the parent material. Analytical procedures were developed to determine mass concentration and oligomer distribution in both the vapor and aerosol phase as well as to determine oligomer distribution as a function of aerosol particle size.

#### **INTRODUCTION**

Information regarding the characterization of physicochemically complex inhalation exposure atmospheres is relatively scarce. The need to conduct a series of experiments to determine the inhalation toxicity, including a no-effect level, of CTFE oligomer-based hydraulic fluids provided the impetus for the development of generation and analytical methods to characterize these chemically and physically complex exposure atmospheres. Two studies were conducted that required the exposure of animals to CTFE atmospheres spanning a 100-fold difference in mass concentration. Exposure concentrations for the two studies were 1.0, 0.5, and 0.25 mg/L, and 0.25, 0.05, and 0.01 mg/L, respectively. Two separate lots of CTFE (3.1 oil) were provided for the studies, both of which consisted primarily of oligomers with carbon chain lengths ranging from C5 to C9. However, approximately 5% of the material used to generate the higher concentration set of exposure atmospheres consisted of oligomers longer than C9.

Simple vaporization methods resulted in extensive fractional distillation of the materials, particularly with regard to the material used to generate higher concentration atmospheres. Because the exposures were to be chemically equivalent to the supply CTFE, it was necessary to use differing approaches to atmosphere generation for the two studies. For the investigation requiring higher concentrations compressed air nebulization techniques were employed. As a result, atmospheres could be generated that included less volatile, long-chain oligomers found in the parent CTFE. Lower

concentrations could be generated without significant fractionation or distillation of the parent material using metered material input, thermally controlled evaporation techniques.

The exposure atmospheres generated for the high concentration investigation were heterogeneous, consisting of both vapor and aerosol - each composed of multiple oligomers. The total CTFE mass concentration of these atmospheres was determined by combining the results of continuous infrared (IR) absorption analysis of the vapor phase with the results of gravimetric analyses of the aerosol. The particle size distribution of the aerosol was determined by cascade impaction. Chromatographic analysis of the vapor oligomer constituents, as well as analyses of vapor plus aerosol oligomer constituents, demonstrated a remarkable identity between exposure atmospheres and parent material. In addition, comparisons were made of the oligomeric constituency of individual particle sizes within the aerosol size distribution.

The exposure atmospheres generated for the low concentration investigation were all monophasic consisting entirely of CTFE vapor. Although both investigations had a 0.25-mg/L concentration group the absence of oligomers longer than C9 in the parent CTFE oil used for the low-concentration study minimized the development of condensation aerosol in exposures conducted at this concentration. However, analysis of the oligomer constituents of the 0.25 mg/L atmospheres did reveal a slight distillation effect.

## MATERIALS AND METHODS

### *Test Material Characterization*

Both lots of CTFE used in these investigations were obtained commercially (Halocarbon Corporation, Hackensack, NJ) and were designated MLO 87-124 and MLO 87-347 for the high- and low-concentration studies, respectively. Both lots of CTFE consisted of a mixture of oligomers with the general formula  $\text{Cl}(\text{CF}_2\text{CFCl})_n\text{Cl}$ , which were refined from crude halocarbon oil formed by the polymerization of CTFE monomer. In the process multiple oligomers are formed. Although even-numbered carbon chains are favored, some odd-numbered carbon chains are formed. Gas chromatographic separation using electron capture detection (GC-ECD) demonstrated that both lots of CTFE had between 12 and 14 separate peaks that contributed a minimum of 0.7% of the total mass. In both lots the majority of mass was found to be C6 and C8 oligomers.

Quality assurance tests were performed on all containers of the test materials prior to their use in the investigations to ensure chemical equivalency. Representative samples obtained from each container were analyzed using IR spectrophotometry (Acculab 4, Beckman Instruments Inc., Fullerton, CA). Figure 3.7-1a and 3.7-1b are typical IR spectra for the two lots of CTFE used. Because the IR absorption of CTFE was dominated by the fluorine-carbon bonds, the response of different oligomer mixtures was essentially identical. The lack of C-H, C=O, and ring-type absorption bands demonstrated the purity of the material. Additional quality assurance tests were performed on all

supply containers using a GC-ECD. Results of the chromatographic method indicated minor differences in component distribution between the two lots of CTFE.

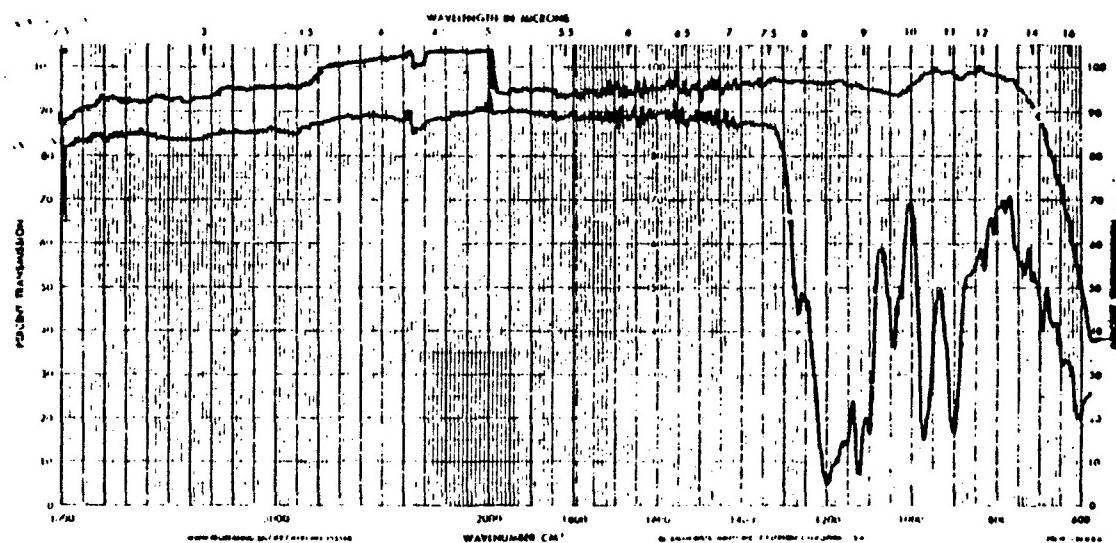


Figure 3.7-1a. Infrared Spectrum of Chlorotrifluoroethylene Oligomer MLO-87-347 (Monophasic Atmospheres).

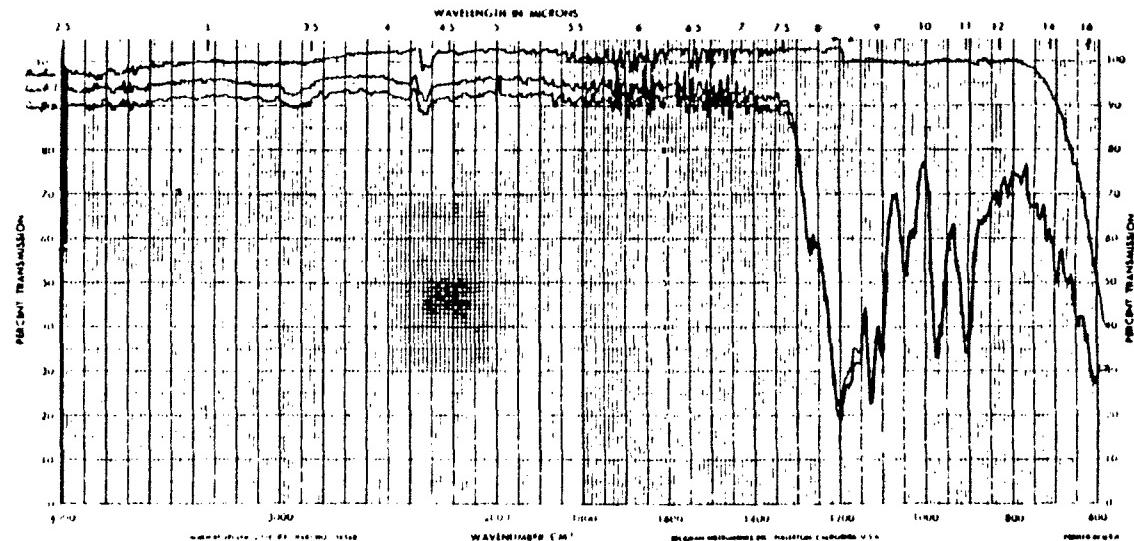


Figure 3.7-1b. Infrared Spectrum of Chlorotrifluoroethylene Oligomer MLO-87-124 (Biphasic Atmospheres).

#### Exposure Atmosphere Generation

As noted above, two methods of atmosphere generation were employed in these investigations. Monophasic, lower concentration atmospheres of MLO 87-347 CTFE were generated

via controlled-evaporation techniques. The CTFE was metered by syringe pump (Sage Model 335, Orion Instruments, Boston, MA) into a 1.85-m, 0.51-cm bore stainless steel tubing evaporator coil. Generator carrier air flow (20 L/min) at room temperature was sufficient to vaporize nearly all the material required to produce target exposure concentrations. However to ensure rapid, complete vaporization of CTFE oligomers with low vapor pressure the evaporator coil was heated to 150 °C.

Generation of higher concentration atmospheres of MLO 87-124 CTFE was accomplished using compressed air nebulizers (Collison, BGI, Waltham, MA). Nebulizers suspended in minimal volume continuous-feed reservoirs produced a mixture of aerosol droplets suspended in saturated vapor. Nebulizer mass output was controlled by varying the number of operational jets as well as the applied pressure. The exposure chamber atmospheres produced following dilution of the nebulizer air flow by the inlet air stream were approximately 90 to 95% vapor, depending on concentration. Rapid equilibration was the result of the relatively high vapor pressure of the low molecular weight oligomers and the extremely large surface area for vaporization presented by an aerosol. Figure 3.7-2a and 3.7-2b are schematic representations of typical generation systems used to produce monophasic and biphasic CTFE atmospheres, respectively.

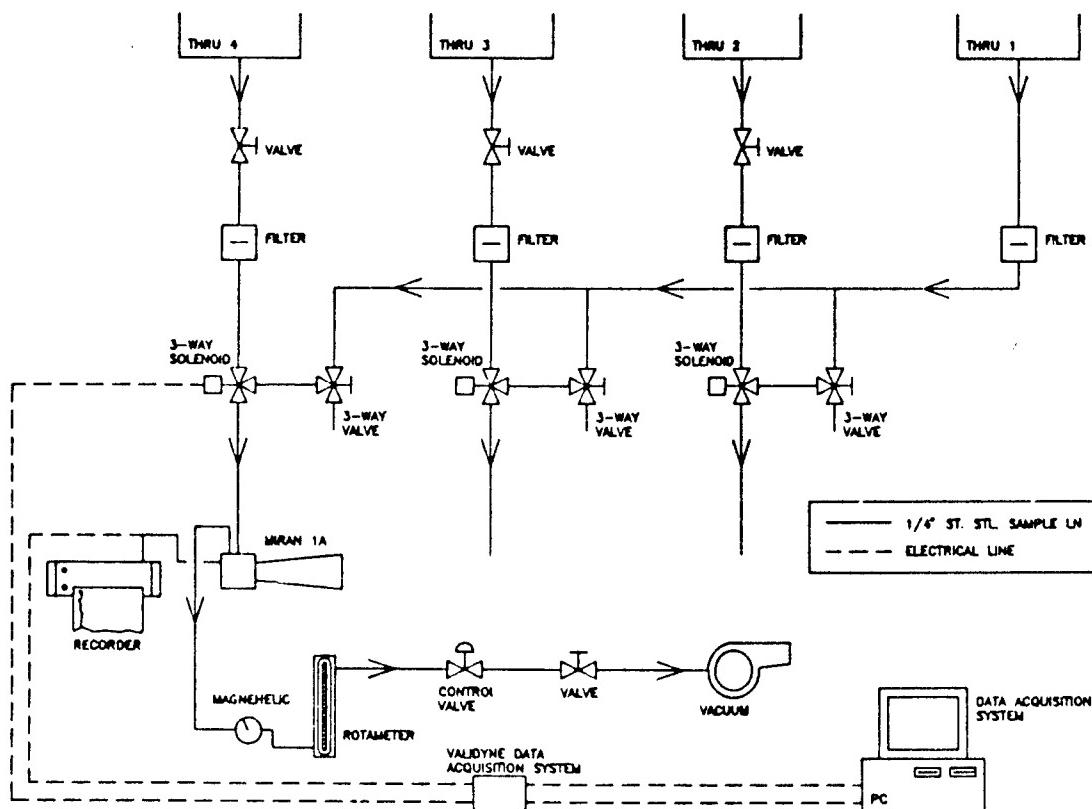
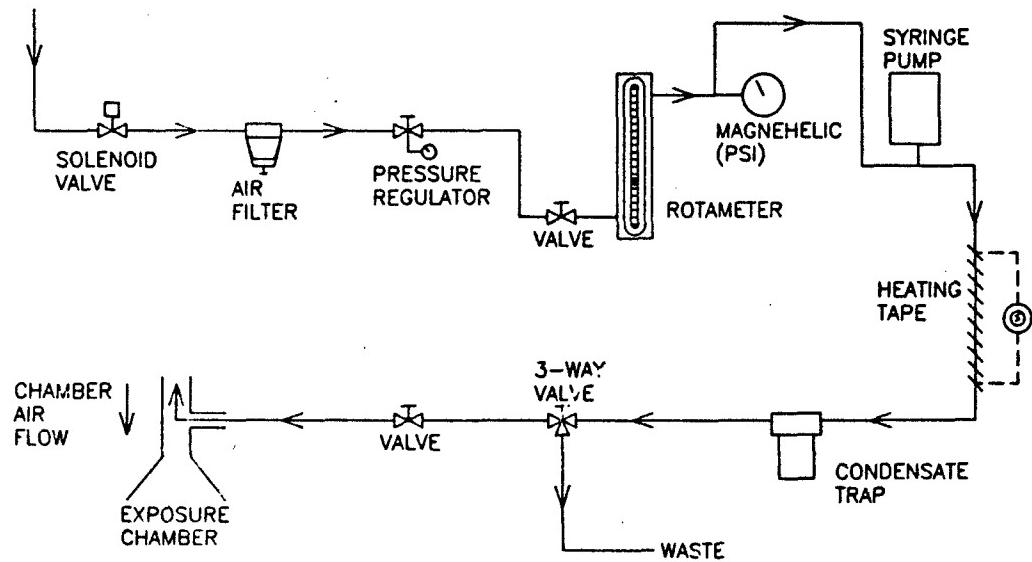


Figure 3.7-2a. Generation System for Monophasic CTFE Atmospheres.

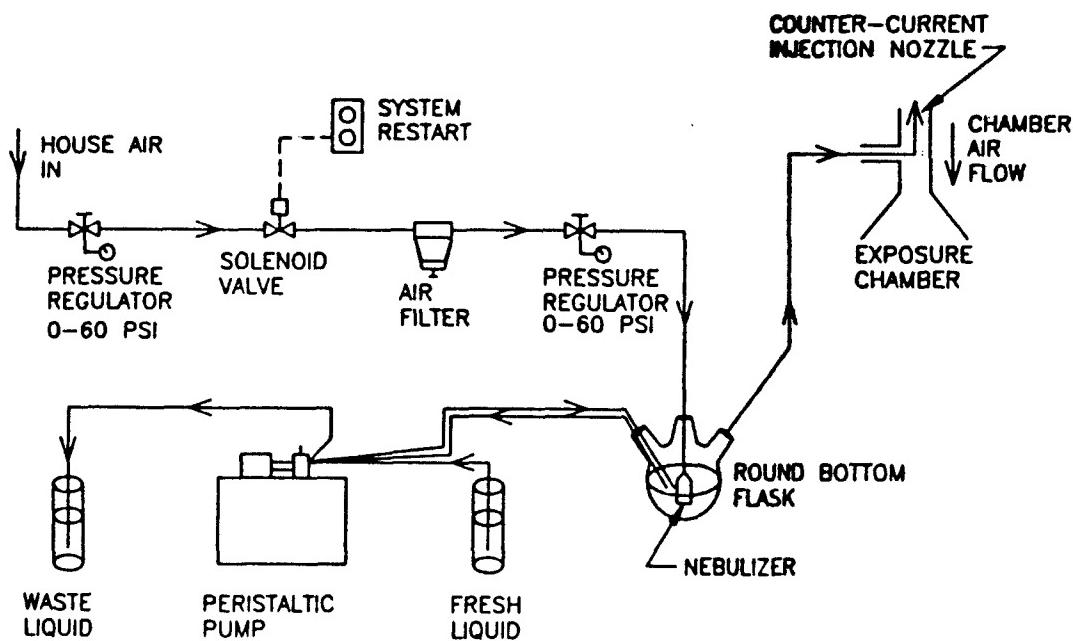


**Figure 3.7-2b. Generation System for Biphasic CTFE Atmospheres.**

The exposure atmospheres were transported from the generators into 690-L Hinners-type inhalation exposure chambers. Generator output was injected counter current to chamber inlet flow and the chambers were equipped with an annular inlet orifice; the combination of countercurrent injection and mixing in a circular, continuous-opposing jet ensured thorough distribution of the CTFE in the exposure chamber flow. Total chamber flow was approximately 185 L/min corresponding to about 13 chamber air exchanges per hour.

#### **Quantitative Analysis of Exposure Atmospheres**

IR absorbance (Miran 1A, Foxboro Instruments, South Norwalk, CT) at 8.3 to 8.5  $\mu\text{m}$  wavelength was used to determine the mass concentration of CTFE vapor in both the monophasic and biphasic atmospheres. Separate spectrometers were used to monitor each exposure concentration on a quasi-continuous basis. Automated, periodic switching of spectrometer sample flow to a zero CTFE concentration atmosphere provided for on-line monitoring and adjustment of spectrometer base line. All sample lines for the spectrometers consisted of 0.51-cm bore stainless steel tubing equipped with in-line filters to remove particulate material. Sampling probes were located in the center of the exposure volume, perpendicular to the chamber bulk flow axis. Vapor mass concentration data, automated sample switching, and adjunct exposure chamber environmental data (relative humidity, pressure, flow, and temperature) were collected using a microprocessor-based data acquisition system (Figure 3.7-3). Periodic calibration of IR instrumentation was performed using standard bag preparation methods; changes of instrument response of greater than  $\pm 5\%$  of target value concentration were cause for adjustment of data acquisition system parameters.



**Figure 3.7-3.** Analytical System for Mass Concentration of CTFE Vapor.

In monophasic CTFE atmosphere studies nephelometers (RAM-S, MEI, Bedford, MA) were incorporated into the analytical system to determine the occurrence of aerosol formation.

Mass concentration of biphasic CTFE exposure atmospheres were determined by addition of IR measurement of the vapor concentration to gravimetric measurement of particle accumulation on glass fiber filters. All filter samples were weighed immediately after collection. Periodically filter samples were reweighed after several hours to determine if there was significant material loss due to evaporation from particle surfaces. A secondary determination of aerosol mass was performed by chromatographic analysis of material gathered on filters. Aerosol size distribution was determined with a modified version of a Lovelace 28-L/min, eight-stage, multijet cascade impactor (Intox Products, Albuquerque, NM).

#### **Chemical Characterization**

Analysis of the oligomer constituency of both lots of parent CTFE and of the exposure atmospheres was performed periodically throughout the investigations. The chromatograph used for both high and low concentration studies was a Varian 3500 equipped with an auto sampler and an ECD. The column was a 30-m wide-bore (0.75 mm), SPB-1 bonded-glass column. All injections were splitless and 1  $\mu$ L in volume. Purified nitrogen was used as the carrier gas at a flow of 6 mL/min, and for make-up gas at the detector at 20 mL/min. Slight variations of detector and column temperatures were used to attempt to separate poorly resolved peaks at the lower concentrations. Injector temperature for both concentration sets was 250 °C; whereas, detector temperature was

320 or 350 °C for the high and low concentrations, respectively. Column temperature programs were (1) 55 °C for 1 min, 15 °C/min to 115 °C, and 5 °C/min to 220 °C and held for the high concentration set, and (2) 50 °C for 1 min, 4 °C/min to 150 °C, and 10 °C/min to 270 °C for the low concentration set.

Samples for the determination of oligomer content of the vapor phase of the exposure atmospheres were collected in a gas-tight syringe from ports located in the sample train upstream from in-line filters. The samples then were dissolved in hexane and the concentration was adjusted to 1 µg/mL. Concentration adjustment was performed to facilitate comparison with the appropriate stock CTFE.

Samples for the determination of oligomer constituency of the aerosol fraction of high-concentration atmospheres were obtained by elution of CTFE from filters obtained for gravimetric analysis. Similarly, hexane elution of CTFE from individual impactor stage substrates was used to determine oligomer constituency of the various particle size fractions. As noted above, all samples were adjusted to 1 µg/mL prior to analysis. Samples for chromatographic analyses of particle mass and oligomer constituency were collected on a weekly basis due to both the large number of samples required and the lengthy processing time for these samples.

## RESULTS

### Exposure Atmosphere Mass Concentration

For both investigations actual CTFE concentration in the exposure atmospheres was remarkably close to the target values with minimal daily variation (Tables 3.7-1 and 3.7-2). Note that two distinct 0.25-mg/L monophasic exposure atmospheres were generated for one investigation. The largest overall deviation from target concentration was 4% at the 0.50-mg/L concentration and the largest coefficient of variation for any of the actual exposure concentrations was 5.7%. As expected, the relative contribution of the aerosol component to the total concentration of the biphasic atmospheres was in direct proportion to total concentration, ranging from 5.3 to 8.8% at 0.25 to 1.0 mg/L, respectively. Nephelometer analysis of the monophasic exposure atmospheres demonstrated the absence of aerosol at any concentration.

TABLE 3.7-1. MASS CONCENTRATION OF MONOPHASIC EXPOSURE ATMOSPHERES

Target concentration	0.25 mg/L	0.25 mg/L	0.05 mg/L	0.01 mg/L
N (exposure days)	64	64	64	64
Total concentration (mg/L)	0.252	0.253	0.049	0.010
Standard deviation	0.0059	0.0052	0.0020	0.0004
CV (%) <sup>a</sup>	2.3	2.1	4.1	4.4

N = Number of exposure days.  
CV = Coefficient of variation.

TABLE 3.7-2. MASS CONCENTRATION OF BIPHASIC EXPOSURE ATMOSPHERES

Target concentration	1.0 mg/L	0.50 mg/L	0.25 mg/L
N (exposure days)	66	66	66
Total concentration (mg/L)	0.98	0.48	0.25
Standard deviation	0.002	0.003	0.004
CV (%)	3.2	4.3	5.7
% Aerosol	8.8	6.8	5.3
MMAD ( $\mu\text{m}$ )	1.14	0.97	1.20
og	2.17	2.15	2.59

N = Number of exposure days.

CV = Coefficient of variation.

MMAD = Mass median aerodynamic diameter.

og = Geometric standard deviation.

#### Exposure Atmosphere Oligomer Distribution

For ease of discussion, individual oligomer peaks on the chromatograms have been separated into two groups. Group 1 peaks include those that eluted between 10 and 15 min and correspond to lower molecular weight oligomers with carbon chain lengths of C5 to C7. Group 2 peaks are those that eluted after 15 min and correspond to higher molecular weight oligomers, which include the remaining C7 to C9 chain length oligomers. Analyses of the exposure atmospheres demonstrated that the more volatile Group 1 oligomers were distributed primarily in the vapor phase, whereas less volatile Group 2 oligomers were distributed in both the vapor and aerosol phases.

Although the overall distribution of oligomers in the monophasic exposure atmospheres was quite similar to that in the parent CTFE, as shown in Table 3.7-3, some subtle differences were observed. Deviation of oligomer distribution from that of the stock CTFE was directly proportional to atmosphere concentration; that is, the higher the concentration the greater the deviation from stock CTFE of individual oligomer contribution to total mass. As exposure concentration increased there was an enrichment of Group 1 oligomers accompanied by a corresponding depletion of the contribution to total mass of Group 2 oligomers. In the worst case there was a 7.1% increase (35.6 vs. 28.5%; exposure atmosphere vs. stock CTFE) in the relative contribution to total mass of the oligomer eluting at 11.8 min in one of the 0.25-mg/L exposure atmospheres. Correspondingly, there was a 6.6% (16.5 vs. 23.1%) decrease in the relative contribution to total mass of the oligomer eluting at 21.3 min in one of the 0.25-mg/L exposure atmospheres.

As observed for monophasic CTFE atmospheres, there was remarkable similarity of overall oligomer distribution in the biphasic exposure atmospheres and the stock CTFE, with some minor shifts of distribution as a function of concentration. As exposure concentration increased, there was an enrichment of lower molecular weight Group 1 oligomer contribution to total mass with a corresponding decrease in the relative contribution of Group 2 oligomers. Figure 3.7-4 illustrates this concentration-driven change in oligomer distribution for the total atmosphere (vapor and aerosol),

and Figure 3.7-5 shows the oligomer distribution shift as a function of exposure concentration in the vapor phase only. Comparison of these figures demonstrates identical oligomer shifts irrespective of monophasic vs. biphasic state. In the worst case the magnitude of the shifts is approximately 5% at the highest concentration as seen for the increase in relative contribution of the oligomer eluting at 11.9 min with a corresponding decrease in the oligomer eluting at 18.7 min. Figures 3.7-6, 3.7-7, and 3.7-8 show that for each exposure concentration the oligomer distribution of the combined vapor and aerosol phases more closely approximates the distribution of oligomers in the stock CTFE than does the oligomer distribution in the vapor phase alone, despite the fact that the aerosol fraction of the exposure concentrations was never greater than 9% of the total.

TABLE 3.7-3. OLIGOMER DISTRIBUTION IN MONOPHASIC ATMOSPHERES

Retention Time	Stock CTFE	Atmosphere Concentration			
		0.25 mg/L	0.25 mg/L	0.05 mg/L	0.01 mg/L
10.2	1.6 ± 0.18	2.0 ± 0.26	2.1 ± 0.31	1.6 ± 0.25	1.6 ± 0.19
11.8	28.5 ± 1.06	33.7 ± 2.26	35.6 ± 2.10	28.4 ± 2.36	29.2 ± 3.00
12.0	8.0 ± 0.51	9.3 ± 0.60	10.0 ± 0.73	8.6 ± 1.30	8.7 ± 0.72
12.8	1.4 ± 0.15	1.3 ± 0.41	1.5 ± 0.46	1.4 ± 0.26	1.2 ± 0.15
13.4	8.0 ± 0.51	9.1 ± 0.55	9.6 ± 0.64	8.1 ± 0.53	8.3 ± 0.46
13.7	4.1 ± 0.20	4.7 ± 0.42	4.9 ± 0.24	4.1 ± 0.33	4.3 ± 0.31
19.4	0.7 ± 0.17	0.7 ± 0.14	0.7 ± 0.22	0.7 ± 0.12	0.7 ± 0.15
19.8	12.7 ± 0.42	12.1 ± 0.61	1.7 ± 0.77	12.4 ± 0.76	12.8 ± 1.86
21.0	3.0 ± 0.36	2.3 ± 0.39	2.0 ± 0.21	2.7 ± 0.87	2.7 ± 0.75
21.3	23.1 ± 0.95	18.8 ± 1.54	16.5 ± 2.07	22.7 ± 2.23	22.9 ± 0.95
22.6	7.2 ± 0.61	5.5 ± 0.67	4.6 ± 0.64	7.0 ± 0.64	7.0 ± 1.69
22.8	1.8 ± 0.48	1.0 ± 0.34	0.7 ± 0.24	1.9 ± 0.71	1.7 ± 0.97
23.8	1.1 ± 0.42	1.0 ± 0.52	1.1 ± 0.44	0.9 ± 0.32	1.0 ± 0.36

Retention time = minutes.

All data = mean percent total mass ± standard deviation.

Initially a discrepancy between gravimetric measurement and chromatographic determination of aerosol mass was noted. Additional IR spectrometric analyses of residues remaining on impactor substrates and from samples of parent CTFE after near complete vaporization evinced additional CTFE oligomers that did not elute in the original chromatographic analysis. These oligomers most probably had carbon chain lengths beyond C9 and were found to account for approximately 5% of the total mass of the stock CTFE. Adjusting for the mass of these oligomers, the discrepancy between gravimetric and chromatographic determination of aerosol was resolved. Additional calculations showed that the contribution of these longer chain oligomers to total mass in the biphasic atmospheres ranged from 4.5 to 5.9% for the 0.25- to 1.0-mg/L exposure concentrations. Although found predominantly in the aerosol phase, the contribution of the longer chain oligomers to total atmosphere concentration was comparable to their concentration in the stock CTFE.

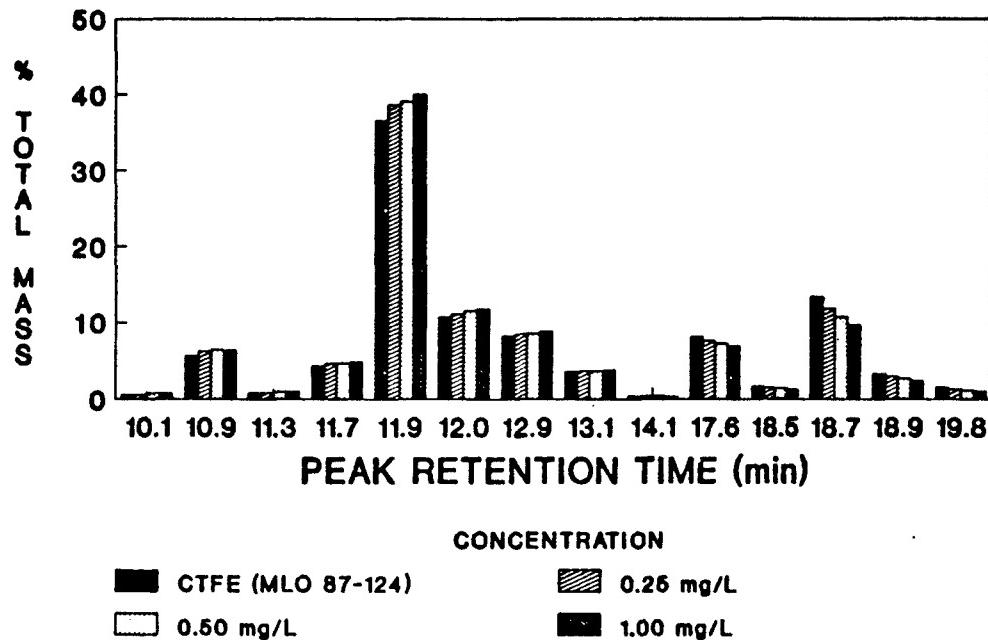


Figure 3.7-4. Oligomer Distribution in CTFE Stock and Biphasic Atmospheres (Vapor and Aerosol).

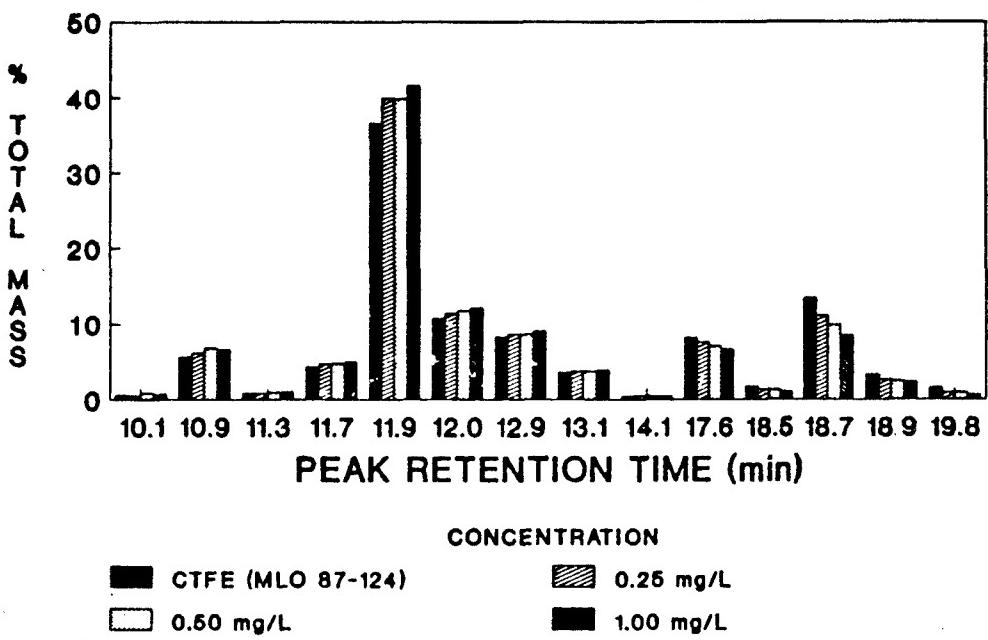


Figure 3.7-5. Oligomer Distribution in CTFE Stock and Biphasic Atmospheres (Vapor Only).

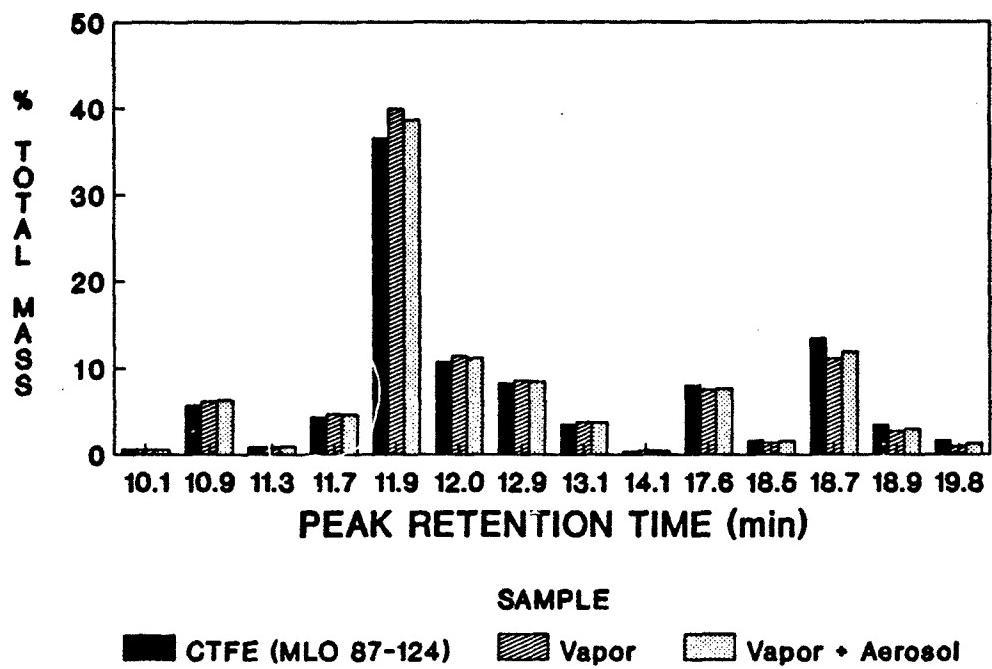


Figure 3.7-6. Oligomer Distribution in CTFE Stock and the 0.25-mg/L Biphasic Atmosphere.

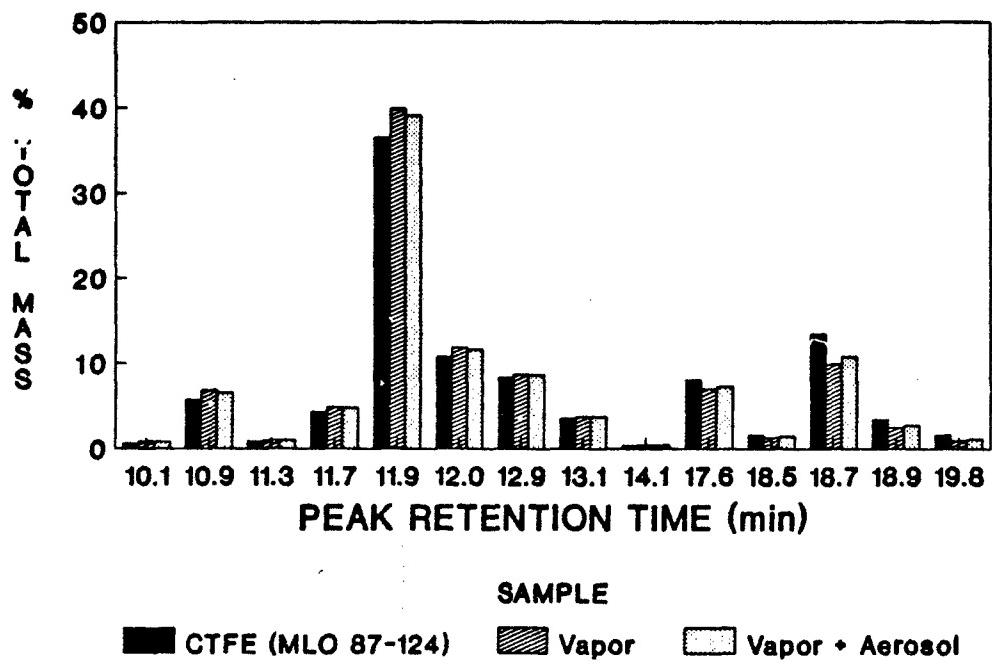


Figure 3.7-7. Oligomer Distribution in CTFE Stock and the 0.50-mg/L Biphasic Atmosphere.

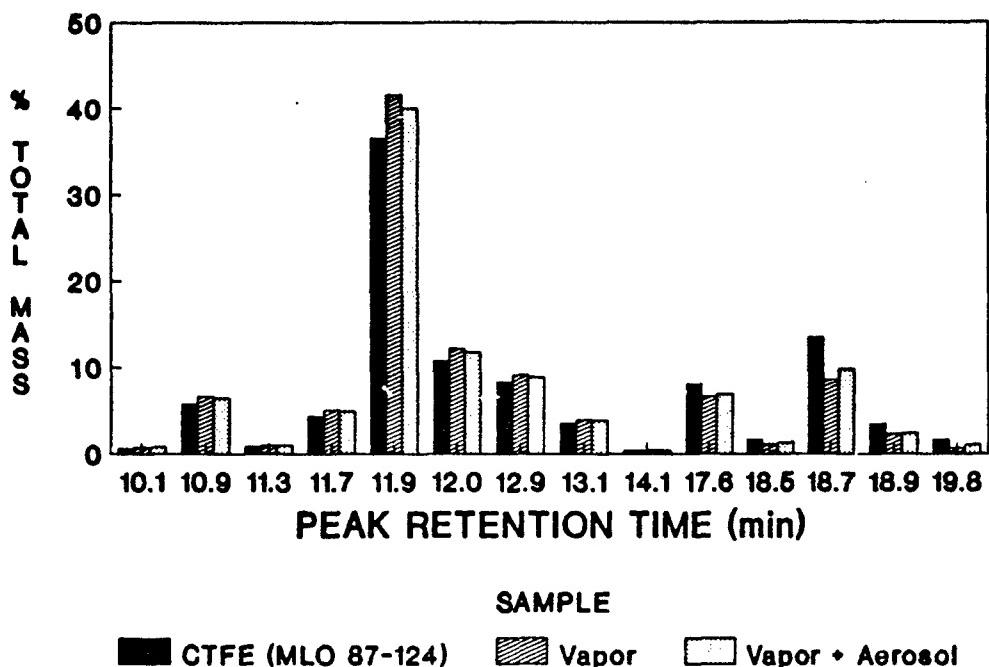


Figure 3.7-8. Oligomer Distribution in CTFE Stock and the 1.00-mg/L Biphasic Atmosphere.

Analysis of oligomer distribution in the aerosol phase alone (Figure 3.7-9) demonstrated that compared to parent CTFE, the aerosol fraction was composed almost entirely of Group 2 and noneluting oligomers. For example, the oligomer eluting at 18.7 min contributed approximately 12% to the total mass of the stock CTFE, whereas it contributed approximately 47% of the identified aerosol mass of the lowest exposure concentration (0.25 mg/L). The distribution of the few Group 1 oligomers present in the aerosol phase did not appear to change as a function of exposure concentration. However, as exposure concentration increased there was a corresponding decrease in the relative contribution to aerosol, hence total, mass by Group 2 oligomers.

Regardless of exposure concentration, the mass median aerodynamic diameter of the aerosols in the biphasic atmospheres was quite similar (Table 3.7-2) being 1.2, 0.97, and 1.14  $\mu\text{m}$  for 0.25-, 0.50-, and 1.0-mg/L concentrations, respectively. The aerosols also were polydisperse irrespective of exposure concentration, having mean micrograms of 2.5, 2.15, and 2.17 for 0.25-, 0.50-, and 1.0-mg/L concentrations, respectively. Consequently, a comparison of oligomer distribution as a function of particle size was made by analyzing individual impactor stage substrates. As shown in Figure 3.7-10 the majority of aerosol mass was collected on impactor stages 5 through 7 corresponding to particle diameters of 1.6, 0.9, and 0.6  $\mu\text{m}$ , respectively. Material collection on other impactor stages was insufficient to produce well-resolved chromatograms, thus only these three particle sizes were analyzed for oligomer distribution. Oligomer distribution was found to be similar in particles of different sizes. At a given exposure concentration oligomer distribution did not vary as a function of individual particle size (Figures 3.7-11 through 3.7-13). However, in particles of a given size, oligomer

distribution varied as a function of exposure concentration. The relative contribution of higher molecular weight oligomers to total mass contributed by a given size particle diminished as exposure concentration increased (Figures 3.7-14 through 3.7-16). This trend was observed in all three discrete particle sizes examined.

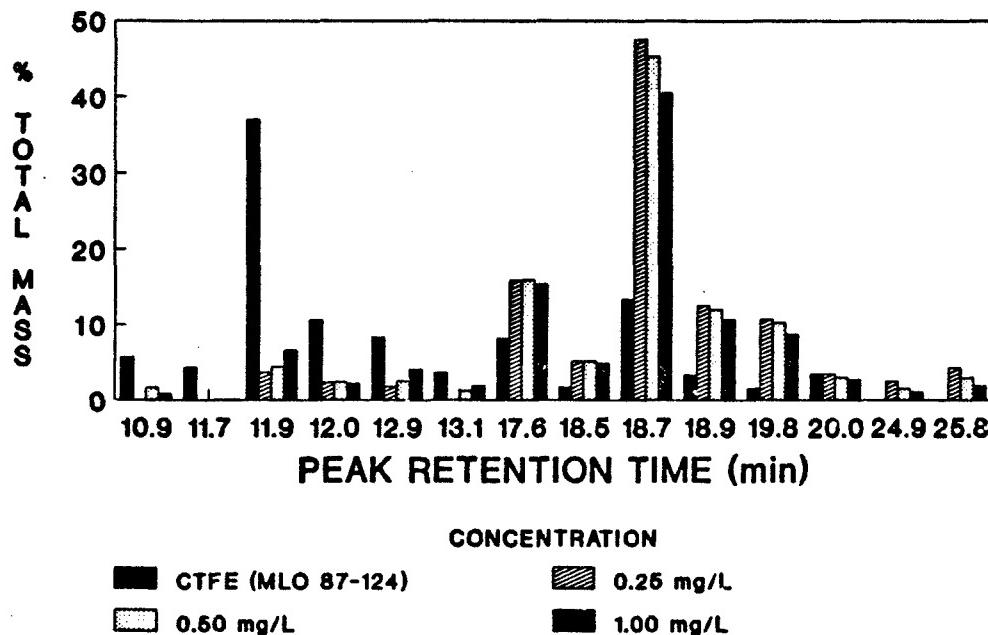


Figure 3.7-9. Oligomer Distribution in CTFE Stock and the Aerosol Fraction of Biphasic Atmospheres.

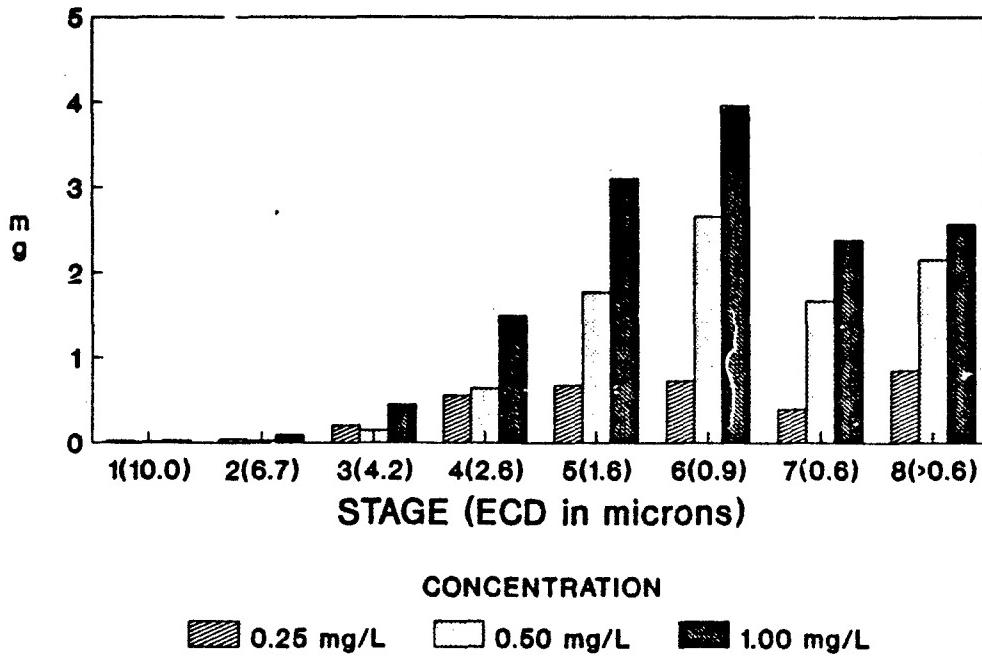


Figure 3.7-10. Aerosol Mass by Impactor Stage (Particle Size). ECD = 50% effective cutoff diameter.

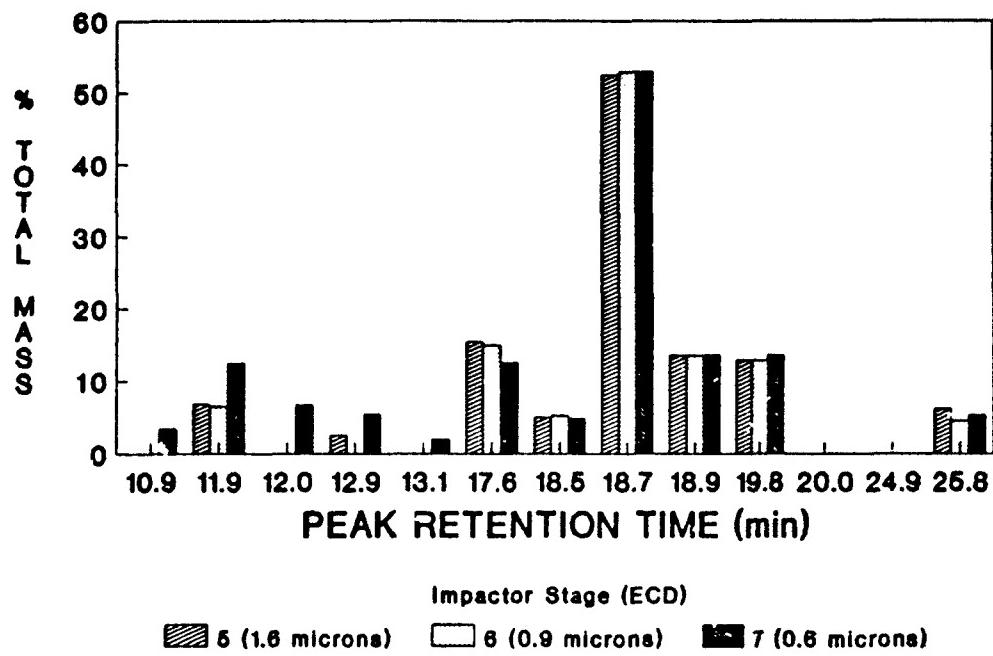


Figure 3.7-11. Oligomer Distribution as a Function of Particle Size in 0.25-mg/L Atmospheres.  
ECD = 50% effective cutoff diameter.

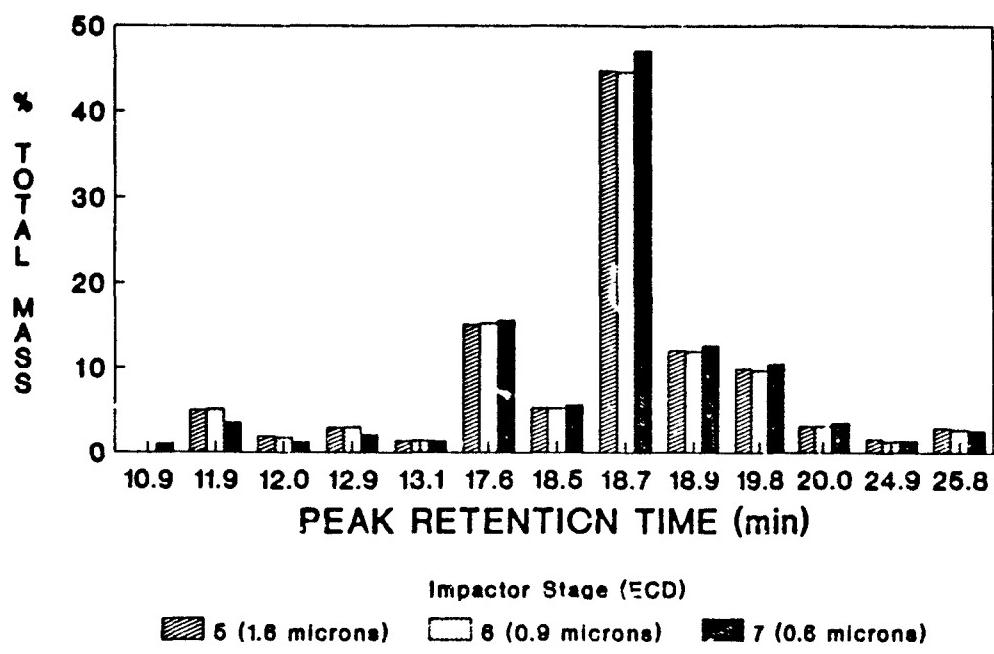


Figure 3.7-12. Oligomer Distribution as a Function of Particle Size in 0.50-mg/L Atmospheres.  
ECD = 50% effective cutoff diameter.

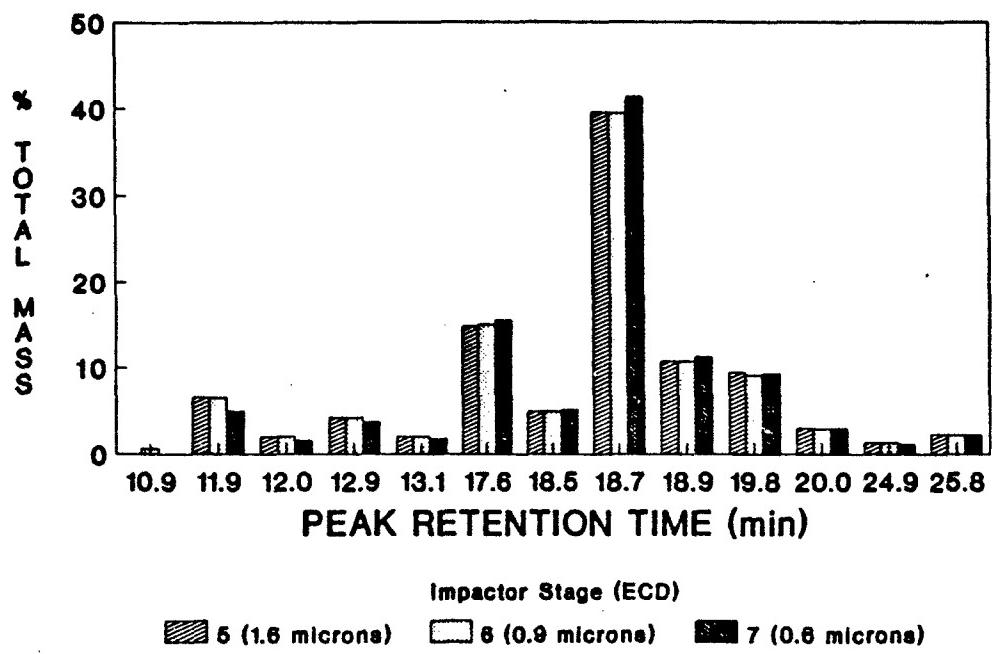


Figure 3.7-13. Oligomer Distribution as a Function of Particle Size in 1.0-mg/L Atmospheres.  
ECD = 50% effective cutoff diameter.

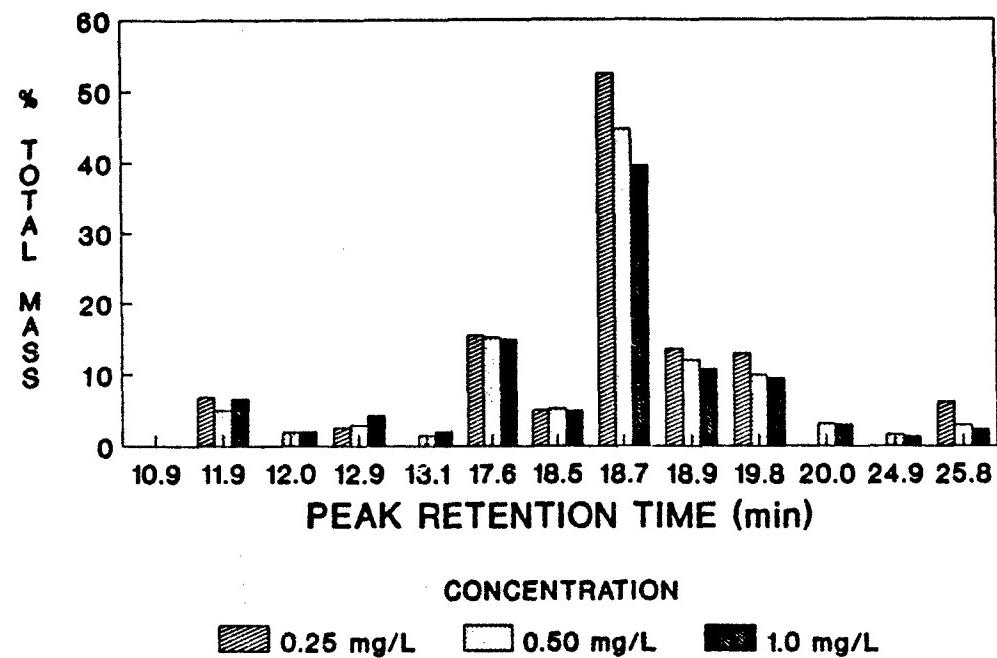


Figure 3.7-14. Oligomer Distribution in 1.6- $\mu\text{m}$  Aerosol Particles as a Function of Atmosphere Concentration. ECD = 50% effective cutoff diameter.

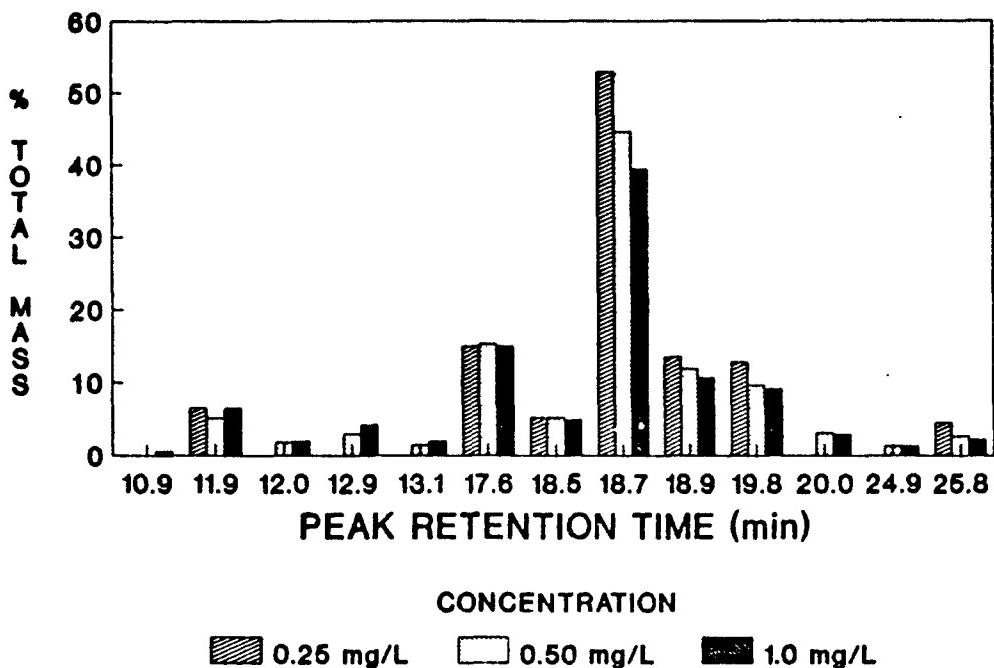


Figure 3.7-15. Oligomer Distribution in 0.9- $\mu\text{m}$  Aerosol Particles as a Function of Atmosphere Concentration. ECD = 50% effective cutoff diameter.

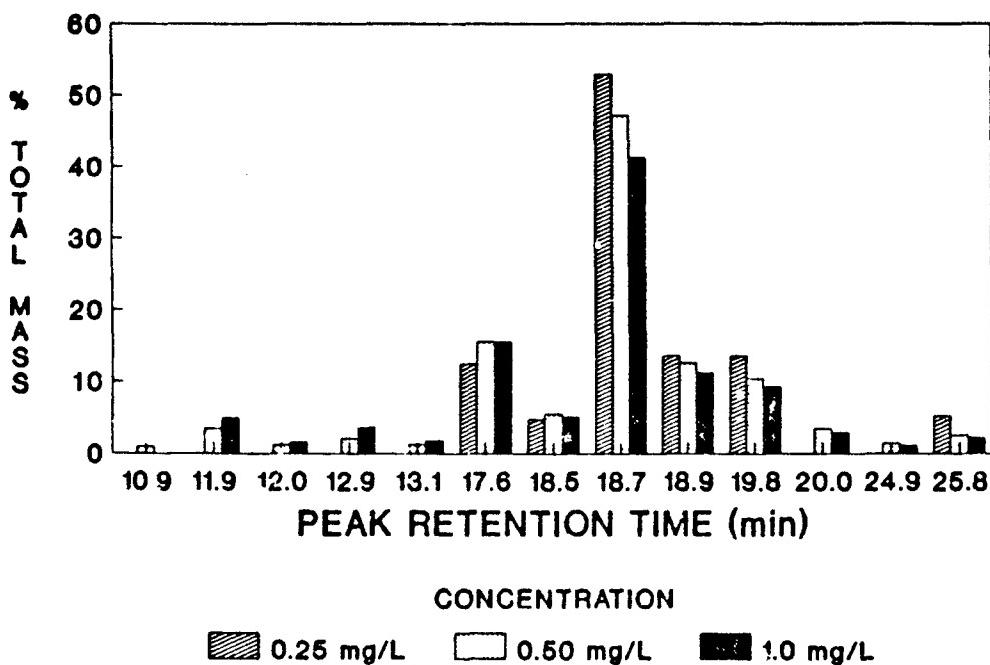


Figure 3.7-16. Oligomer Distribution in 0.6- $\mu\text{m}$  Aerosol Particles as a Function of Atmosphere Concentration. ECD = 50% effective cutoff diameter.

## **DISCUSSION**

Stable, integrated generation and analytical systems and methods have been developed for consistent, reproducible generation and characterization of physicochemically complex inhalation exposure atmospheres of CTFE oligomers ranging three orders of magnitude in concentration. Daily variation in all exposure concentrations was minimal (an average of 3.6%) and it was only slightly larger than what the analysis of system measurement and calibration error would account for. Although minor changes in chemical constituency were found between exposure atmospheres and parent CTFE as a function of exposure concentration, there was a remarkable overall similarity of the chemical composition of the parent material and the exposure atmospheres. The results of in-depth characterization of the exposure atmospheres from two investigations using different lots of CTFE demonstrate a reasonable chemical continuity of the exposure atmospheres from one study to the next despite differences in generation methodology and source material. This relative chemical continuity between the exposure atmospheres facilitates comparisons of the relative toxicity of CTFE observed in these investigations.

### **3.8 LD<sub>50</sub> AND LD<sub>10</sub> ORAL TOXICITY STUDIES OF CHLOROTRIFLUOROETHYLENE ACIDS IN F-344 RATS**

**E.R. Kinkead, S.K. Bunger, and R.E. Wolfe**

#### **ABSTRACT**

Chlorotrifluoroethylene (CTFE) is a hydraulic fluid that consists mostly of C6 and C8 oligomers. The C8 (tetramer) oligomers accumulate preferentially during long-term exposure and appear to be more hepatotoxic than the C6 (trimer) oligomers. To aid in further development of a pharmacokinetic model and to understand the contribution of each acid group, male and female rats were gavaged with a single dose equivalent of the LD<sub>10</sub> dose of each acid and maintained for periods of up to 14 days. Body fluids, feces, and tissues were collected at varying time points for CTFE acid and metabolite analysis. Relative liver and kidney weights were increased in tetramer-treated but not the trimer-treated rats.

#### **INTRODUCTION**

Chlorotrifluoroethylene (halocarbon 3.1 oil) oligomer is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectric strength. A recently concluded 90-day inhalation study to 1000, 500, and 250 mg CTFE/m<sup>3</sup> in this laboratory resulted in a dose-dependent depression in body weight gains of male rats (Kinkead et al., 1989). Alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase values examined at the conclusion of the study indicated a treatment-response effect in the male test rats but not in the female test rats. Relative kidney and liver weights of the male test rats were elevated significantly when compared to the respective control groups. The primary target organ of CTFE was the liver. Gross liver hypertrophy and microscopic hepatocytomegaly were the principal manifestations of CTFE-induced hepatotoxicity. The liver pathology was similar to that seen following acute exposure to perfluorodecanoic acid. This suggests that CTFE toxicity may be due to the formation of acid metabolites. Halocarbon 3.1 oil is an isomeric mixture of trimeric and tetrameric oligomers that, when chromatographed, results in two distinct groups of peaks. The contributions of CTFE trimer acid (corresponding to the first set of peaks) and tetramer acid (corresponding to the second set of peaks) to the overall toxicity of CTFE were addressed independently.

The oral toxicity of CTFE trimer and tetramer acids was evaluated in male and female Fischer 344 (F-344) rats. The maximum tolerated repeated oral dose concentration was determined for use in future subchronic or chronic toxicity studies. Data also were collected to support further development of a physiologically based pharmacokinetic model.

## MATERIALS AND METHODS

### *Animals*

Upon receipt from Charles River Breeding Labs, Kingston, NY, all rats were quality control tested prior to use in the studies. The animals were group housed (three per cage) in clear plastic cages with wood-chip bedding. The male and female rats dosed at the LD<sub>10</sub> value were housed in metabolism cages (Nalge Company, Rochester, NY) for a 14-day period. Water and feed (Purina Formulab #5008) were available ad libitum except for 12 h prior to sacrifice. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

### *Test Agent*

The CTFE acid samples used in this study were purchased by the Air Force from Technolube Products, Inc., Ultrasystems, Inc., Irvine, CA. The samples were identified as follows.

CTFE Trimer acid  
ID#10-86-40 IR#14086  
B.P. 82-85C/10-3 mm Hg  
Eq. wt. 357.1 M.W. 363.5

CTFE Tetramer acid  
ID#10-86-44 IR#14132  
B.P. 110-124C/10-3 mm Hg  
Eq. Wt. 474.6 M.W. 480

### *LD<sub>50</sub> and LD<sub>10</sub> Treatment Regimen*

Five animals per sex were treated with appropriately spaced dose levels to produce a range of mortality rates sufficient to permit the determination of LD<sub>50</sub> and LD<sub>10</sub> values. The rats were fasted for approximately 16 h prior to administration of the compound. Dose volumes were maintained at 0.01 mL/g body weight. The test compounds were diluted in corn oil, which previously had been tested for the presence of peroxides. The rats were weighed individually at the time of dosing to determine dose volume.

Records were maintained for body weights. Twenty-four hour urine and fecal samples were collected for metabolite analysis from the LD<sub>10</sub> animals only. Urine samples also were analyzed for inorganic fluoride content. Blood samples were collected via the lateral tail vein and analyzed for CTFE acid and/or metabolites. Tissues (liver, lungs, kidney, muscle, brain, testes, and fat) also were analyzed for CTFE acids and/or metabolites. Sections of liver were prepared for examination by transmission electron microscopy and light microscopy. Kidneys and livers of LD<sub>10</sub> male rats were weighed at all serial sacrifices through seven days.

### ***Analyses of Biological Samples***

Blood for CTFE acid analysis was transferred to a 20-mL scintillation vial containing 5.0 mL of hexane. To reduce CTFE vaporization, the transfer occurred below the hexane surface. CTFE was extracted for 2 h using an Evapotec® mixer (Haake Buchler Instruments, Inc., Saddlebrook, NJ).

To avoid analysis interference, no refrigerant or preservative was used during the collection of 24-h urine samples for either CTFE or fluoride analysis. The samples were transferred into hexane following the same procedures as described for blood. CTFE was extracted using an Evapotec® mixer for 1 h.

Fluoride concentration of the urine was determined by ion-specific electrodes using the method of Neefus (1970). Urine weight was converted to volume using a value of 1.06 g/mL for urine density. Fluoride concentration of the urine was determined by diluting the urine by 50% with a total ionic strength buffer (Neefus et al., 1970) and measuring the resulting cell potential with an Orion 701A (Orion Research, Inc., Cambridge, MA) analyzer. The instrument was calibrated by measuring the cell potential of standard solutions using the same buffer and dilution conditions.

Tissues collected for CTFE acid analysis were weighed and then maintained in hexane on ice until homogenization. The tissues were homogenized using a Tissue-Mizer® (Tekmar, Cincinnati, OH), then mixed overnight on an Evapotec® mixer. Following centrifugation, the hexane layer was stored at -70°C, but allowed to return to room temperature before analysis. Tissue extracts were diluted with hexane to reduce the CTFE in the samples to less than 500 ng/mL for calibration purposes.

Peroxisomal  $\beta$ -oxidation assays were performed on approximately one gram of liver tissue. Samples were removed from the median lobe and placed in ice-cold 0.25 M sucrose. The sample was homogenized, then centrifuged at 1500  $\times$  g for 10 min. A portion of the resultant supernatant was used to measure the rate of palmitoyl coenzyme A oxidation (Lazarow, 1982).

### ***Statistics***

The LD<sub>50</sub> and LD<sub>10</sub> values were calculated using either a probit analysis method (SAS Probit; SAS, 1985) or a logistic regression method (Dixon 1985). Peroxisomal  $\beta$ -oxidation assay values were analyzed via a nonparametric analysis of variance (SAS, 1985).

## ***RESULTS***

### ***LD<sub>50</sub> Assays***

Groups of five male and five female rats were given single peroral doses of the CTFE trimer and tetramer acids and the results are shown in Tables 3.8-1 through 3.8-4. Because the primary purpose of the LD<sub>50</sub> dosing was to determine levels that would cause little mortality (i.e., LD<sub>10</sub> values that would be used for the metabolic assays), no attempt was made to produce tight values and, therefore, 95% confidence limits are not reported. Deaths from the trimer acid occurred within three

days of dosing whereas deaths from the tetramer acid were delayed up to eight days posttreatment. Mean body weights of surviving rats are included in the tables. The only group that demonstrated less than normal weight gain over the 14 days was the group dosed with 99 mg tetramer acid/kg. Transient body weight loss was seen in all groups but generally was reversed by the fourth posttreatment day. Rats that died during the posttreatment observation period had not regained their initial body weight.

TABLE 3.8-1. RESULTS OF ACUTE ORAL DOSING OF CTFE TRIMER ACID TO MALE F-344 RATS

Dose Level (mg/kg)	14-Day Mortality Ratio	Days to Death	Average 14-Day Weight Gain of Survivors (g)
1000	5/5	All at 1	-
500	4/5	2,2,2,3	52
250	1/5	2	62

LD<sub>50</sub> = 396 mg/kg.

LD<sub>10</sub> = 212 mg/kg.

TABLE 3.8-2. RESULTS OF ACUTE ORAL DOSING OF CTFE TRIMER ACID TO FEMALE F-344 RATS

Dose Level (mg/kg)	14-Day Mortality Ratio	Days to Death	Average 14-Day Weight Gain of Survivors (g)
1000	3/5	1,1,2	29
500	3/5	2,2,2	29
250	2/5	2,3	30

LD<sub>50</sub> = 396 mg/kg.

LD<sub>10</sub> = 12 mg/kg.

TABLE 3.8-3. RESULTS OF ACUTE ORAL DOSING OF CTFE TETRAMER ACID TO MALE F-344 RATS

Dose Level (mg/kg)	14-Day Mortality Ratio	Days to Death	Average 14-Day Weight Gain of Survivors (g)
500	5/5	1,1,2,2,3	-
250	5/5	4,4,4,5,5	-
125	5/5	5,5,6,6,8	-
99	1/5	7	11
79	5/5	5,6,6,6,6	-
63	0/5	-	36
31	0/5	-	50

LD<sub>50</sub> = 88 mg/kg.

LD<sub>10</sub> = 53 mg/kg.

TABLE 3.8-4. RESULTS OF ACUTE ORAL DOSING OF CTFE TETRAMER ACID TO FEMALE F-344 RATS

Dose Level (mg/kg)	14-Day Mortality Ratio	Days to Death	Average 14-Day Weight Gain of Survivors (g)
500	5/5	1,2,2,2,2	-
250	0/5	-	33
125	0/5	-	29

$LD_{50} = 352 \text{ mg/kg}$ .  
 $LD_{10} = 200 \text{ mg/kg}$ .

#### LD<sub>10</sub> Assays

Male rats were dosed at the trimer acid and tetramer acid LD<sub>10</sub> concentrations (212 and 53 mg/kg, respectively). Five animals per group were serially sacrificed at 1, 3, 24, and 48 h and 7 days posttreatment. Livers were collected for light and electron microscopy. Sections of liver, kidney, muscle, brain, lung, testis, and fat were taken for CTFE analysis. Analyses of fat, liver, feces, and urine have been initiated.

Livers and kidneys of the five groups of rats were weighed and compared with control values. These data are given in Table 3.8-5. Increased relative liver weights ( $p < 0.05$ ) were noted in the tetramer acid-dosed rats at the seven-day sacrifice. The relative liver weights of the trimer acid rats were increased slightly at both the 48-h and the seven-day sacrifices, but the difference was not statistically significant. Relative kidney weights were increased ( $p < 0.05$ ) in the tetramer acid-dosed group at the seven-day posttreatment period only.

TABLE 3.8-5. LIVER AND KIDNEY WEIGHTS<sup>a</sup> OF MALE F-344 RATS ORALLY DOSED WITH CTFE ACIDS AT THE LD<sub>10</sub> CONCENTRATION LEVEL

	Untreated Control	Corn Oil Control	Tetramer LD <sub>10</sub>	Trimer LD <sub>10</sub>
<b>1 h</b>				
Liver	11.15 ± 0.48	12.03 ± 0.53	9.67 ± 0.41	11.75 ± 0.49
Ratio	4.95 ± 0.18	5.30 ± 0.24	4.51 ± 0.06	5.11 ± 0.26
Kidney	1.88 ± 0.05	1.93 ± 0.06	1.84 ± 0.07	1.94 ± 0.08
Ratio	0.83 ± 0.02	0.85 ± 0.02	0.86 ± 0.02	0.84 ± 0.04
<b>3 h</b>				
Liver	12.12 ± 0.72	12.62 ± 0.72	11.57 ± 0.82	12.04 ± 1.00
Ratio	5.13 ± 0.18	5.59 ± 0.16	5.11 ± 0.27	5.22 ± 0.24
Kidney	2.09 ± 0.10	2.11 ± 0.08	1.94 ± 0.07	2.01 ± 0.08
Ratio	0.89 ± 0.01	0.94 ± 0.01	0.86 ± 0.01	0.88 ± 0.01
<b>24 h</b>				
Liver	11.38 ± 0.44	13.23 ± 0.85	12.83 ± 0.15	12.51 ± 0.47
Ratio	5.29 ± 0.22	5.46 ± 0.25	5.65 ± 0.22	5.69 ± 0.14
Kidney	1.98 ± 0.05	2.16 ± 0.08	2.04 ± 0.03	2.00 ± 0.03
Ratio	0.92 ± 0.02	0.90 ± 0.02	0.90 ± 0.02	0.91 ± 0.02

(continued)

TABLE 3.8-5. Continued

	Untreated Control	Corn Oil Control	Tetramer LD <sub>10</sub>	Trimer LD <sub>10</sub>
<b>48 h</b>				
Liver	14.28 ± 0.20	13.61 ± 0.47	16.48 ± 0.48	15.24 ± 0.51
Ratio	5.94 ± 0.06	5.82 ± 0.09	7.08 ± 0.17	6.42 ± 0.11
Kidney	2.23 ± 0.03	2.17 ± 0.08	2.02 ± 0.18	2.24 ± 0.08
Ratio	0.93 ± 0.01	0.93 ± 0.01	0.87 ± 0.08	0.94 ± 0.01
<b>7 day</b>				
Liver	13.25 ± 0.84	13.14 ± 0.64	15.73 ± 0.34	12.94 ± 0.02
Ratio	5.13 ± 0.28	5.38 ± 0.33	7.80 ± 0.17 <sup>b,c</sup>	5.41 ± 0.28
Kidney	2.18 ± 0.05	2.18 ± 0.06	2.11 ± 0.02	2.21 ± 0.10
Ratio	0.85 ± 0.01	0.89 ± 0.03	1.05 ± 0.01 <sup>b,c</sup>	0.93 ± 0.01

<sup>a</sup> Mean ± SEM, N = 5.<sup>b</sup> Significantly different than corn oil control at p < 0.05 as determined by a two-factorial multivariate analysis of variance with multiple comparisons.<sup>c</sup> Significantly different than untreated control at p < 0.05 as determined by a two-factorial multivariate analysis of variance with multiple comparisons.

Groups of five male and five female rats were dosed at the trimer and tetramer LD<sub>10</sub> concentrations. These animals, along with a similar group of corn oil controls, were maintained in metabolism cages for 14 days posttreatment. Blood samples were collected at 1, 3, 24, and 48 h, and 7 and 14 days posttreatment for CTFE acid metabolite analysis. Daily urine samples were collected for analysis of metabolites and for inorganic fluoride concentrations. Daily fecal samples also were collected for metabolite analyses. The analyses of these biological samples have been initiated.

Daily urine volumes are listed in Tables 3.8-6 and 3.8-7. Urine output of the trimer- and tetramer-dosed groups did not differ significantly from the controls. Inorganic fluoride analysis of these samples is in progress.

TABLE 3.8-6. DAILY URINE OUTPUT<sup>a</sup> OF MALE F-344 RATS ORALLY DOSED WITH CTFE ACIDS AT THE LD<sub>10</sub> CONCENTRATION

Day Posttreatment	Corn Oil Controls	Trimer LD <sub>10</sub>	Tetramer LD <sub>10</sub>
1	7.0 ± 0.5	14.5 ± 0.4 <sup>b</sup>	6.7 ± 0.5
2	7.4 ± 0.6	7.6 ± 0.4	6.8 ± 0.5
3	8.4 ± 0.6	8.8 ± 0.6	7.4 ± 0.2
4	9.0 ± 0.4	9.2 ± 0.6	9.0 ± 0.3
5	8.6 ± 0.3	9.0 ± 0.2	9.1 ± 0.4
6	8.8 ± 0.4	8.6 ± 0.3	8.8 ± 0.5
7	7.7 ± 0.5	7.6 ± 0.4	8.3 ± 0.6 (continued)

TABLE 3.8-6. Continued

Day Posttreatment	Corn Oil Controls	Trimer LD <sub>10</sub>	Tetramer LD <sub>10</sub>
8	9.2 ± 0.4	8.8 ± 0.3	9.0 ± 1.1
9	9.1 ± 0.4	8.7 ± 0.6	9.5 ± 0.4
10	9.1 ± 0.3	8.4 ± 0.4	8.5 ± 0.3
11	8.9 ± 0.7	8.3 ± 0.3	8.6 ± 0.7
12	10.2 ± 0.5	9.0 ± 0.5	9.3 ± 0.4
13	10.9 ± 0.6	9.1 ± 0.4	9.3 ± 0.4
14	17.4 ± 2.8 <sup>c</sup>	19.7 ± 3.7 <sup>d</sup>	15.4 ± 1.6

<sup>a</sup> Mean 24-h sample volume (milliliter) ± SEM, N = 5.<sup>b</sup> Statistically different than control at p < 0.05.<sup>c</sup> N = 4.<sup>d</sup> N = 3.TABLE 3.8-7. DAILY URINE OUTPUT<sup>a</sup> OF FEMALE F-344 RATS ORALLY DOSED WITH CTFE ACIDS AT THE LD<sub>10</sub> CONCENTRATION

Day Posttreatment	Corn Oil Controls	Trimer LD <sub>10</sub>	Tetramer LD <sub>10</sub>
1	6.6 ± 0.3	5.3 ± 0.6	11.9 ± 1.4
2	8.1 ± 0.5	6.8 ± 0.8	7.8 ± 1.9
3	9.4 ± 1.0	8.0 ± 1.0	5.7 ± 1.6
4	11.9 ± 0.7	10.5 ± 0.9	6.6 ± 1.9
5	10.9 ± 1.3	9.4 ± 0.6	7.3 ± 2.4
6	11.2 ± 0.6	10.2 ± 1.2	6.5 ± 2.1
7	12.4 ± 3.2	7.5 ± 0.4	6.2 ± 1.6
8	13.1 ± 3.4	9.6 ± 1.6	7.2 ± 0.7
9	14.4 ± 3.8	10.9 ± 1.5	9.2 ± 0.5
10	15.5 ± 3.0 <sup>b</sup>	10.6 ± 1.7	10.3 ± 0.4
11	11.9 ± 1.5 <sup>c</sup>	9.3 ± 0.7	10.5 ± 0.9
12	12.5 ± 0.8 <sup>c</sup>	8.8 ± 1.7	13.1 ± 2.0
13	12.0 ± 1.7 <sup>c</sup>	9.9 ± 2.0 <sup>b</sup>	14.5 ± 2.1
14	29.8 ± 2.7	17.0 ± 1.5 <sup>b</sup>	14.7 ± 2.0

<sup>a</sup> Mean 24-h sample volume (milliliter) ± SEM, N = 5.<sup>b</sup> N = 4.<sup>c</sup> N = 3.

At sacrifice, all of the above animals plus a similar group of untreated control rats had livers removed and weighed. Sections of liver were sampled for peroxisomal β-oxidation assays, CTFE metabolite analysis, and for light and electron microscopy. In addition to liver, sections of kidney, muscle, brain, testes, lung, and fat were removed from all male rats for CTFE metabolite analysis.

Relative liver weights were increased in male rats treated with the trimer acid LD<sub>10</sub> and in female rats treated with the tetramer acid LD<sub>10</sub> (Table 3.8-8). Although these increases were statistically significant, the increases in relative weight measured 14 days after dosing were less than that seen after the seven-day sacrifice. The results of the peroxisomal  $\beta$ -oxidation assays performed on livers of representative animals from each treatment group are listed in Table 3.8-9. No statistically significant differences were found in the rate of peroxisomal  $\beta$ -oxidation in any of the acid-treatment groups when compared with control rats.

**TABLE 3.8-8. BODY AND LIVER WEIGHTS AND LIVER/BODY WEIGHT RATIOS<sup>a</sup> OF RATS ORALLY DOSED WITH CTFE ACIDS AT THE LD<sub>10</sub> CONCENTRATION AND MAINTAINED 14 DAYS**

	Untreated Control	Corn Oil Control	Trimer LD <sub>10</sub>	Tetramer LD <sub>10</sub>
Males				
<b>Body weights(g)</b>				
0 day	234.1 $\pm$ 2.6	234.3 $\pm$ 2.4	234.1 $\pm$ 3.0	234.2 $\pm$ 4.0
14 day	248.4 $\pm$ 3.3	232.4 $\pm$ 1.8	235.4 $\pm$ 2.9	234.0 $\pm$ 3.8
<b>Liver (g)</b>				
Ratio (%)	9.536 $\pm$ 0.2	7.627 $\pm$ 0.3	8.764 $\pm$ 0.4 <sup>c</sup>	8.206 $\pm$ 0.4
<b>Body weights(g)</b>				
0 day	153.2 $\pm$ 4.0	153.8 $\pm$ 4.7	155.8 $\pm$ 3.6	154.7 $\pm$ 4.8
14 day	153.4 $\pm$ 2.2	150.1 $\pm$ 2.0	154.3 $\pm$ 1.4	147.6 $\pm$ 4.2
<b>Liver(g)</b>				
Ratio(%)	4.938 $\pm$ 0.1	4.904 $\pm$ 0.1	4.832 $\pm$ 0.1	6.383 $\pm$ 0.4
<b>Females</b>				
<b>Body weights(g)</b>				
0 day	153.2 $\pm$ 4.0	153.8 $\pm$ 4.7	155.8 $\pm$ 3.6	154.7 $\pm$ 4.8
14 day	153.4 $\pm$ 2.2	150.1 $\pm$ 2.0	154.3 $\pm$ 1.4	147.6 $\pm$ 4.2
<b>Liver(g)</b>				
Ratio(%)	3.221 $\pm$ 0.1	3.268 $\pm$ <0.1	3.131 $\pm$ <0.1	4.358 $\pm$ 0.3 <sup>c</sup>

<sup>a</sup> Mean  $\pm$  SEM, N = 5.

<sup>b</sup> Fasted weights.

<sup>c</sup> Significantly different than corn oil at p<0.05 using a two-factorial analysis of variance with multivariate comparisons.

<sup>d</sup> Significantly different than corn oil at p<0.01 using a two-factorial analysis of variance with multivariate comparisons.

**TABLE 3.8-9. RATE OF PEROXISOMAL OXIDATION<sup>a</sup> IN RATS ORALLY DOSED WITH CTFE ACIDS AT THE LD<sub>10</sub> CONCENTRATION AND MAINTAINED 14 DAYS**

Treatment	Sex	Enzyme Activity <sup>b</sup>
Corn Oil	M	28.7 $\pm$ 1.1
Corn Oil	F	18.2 $\pm$ 1.4
Untreated	M	15.9 $\pm$ 3.1
Untreated	F	21.1 $\pm$ 1.9
Trimer	M	18.4 $\pm$ 0.7
Trimer	F	13.3 $\pm$ 1.4
Tetramer	M	23.3 $\pm$ 1.7
Tetramer	F	35.4 $\pm$ 8.9

<sup>a</sup> Micromoles/minute/gram liver.

<sup>b</sup> Mean  $\pm$  SEM, N = 3

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### 3.9 REPEATED-DOSE GAVAGE STUDIES ON CHLOROTRIFLUOROETHYLENE ACIDS

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#### ABSTRACT

C<sub>8</sub> chlorotrifluoroethylene (CTFE) oligomers accumulate preferentially during long-term exposure and appear to be more hepatotoxic than C<sub>6</sub> oligomers. A repeated-dose study was initiated to determine the relative contributions of the corresponding C<sub>6</sub> and C<sub>8</sub> acid metabolites to the toxicity of CTFE in the male Fischer 344 rat. Test animals are being dosed once per week for various time periods up to one year. The animals are currently in Week 36 of treatment. A depression ( $p < 0.05$ ) in mean body weight of the highest dose C<sub>8</sub> acid (2.16 mg/kg) group has continued since the second week of the study. An increase in relative liver weight and in hepatic peroxisomal  $\beta$ -oxidation activity was found in the 2.16-mg CTFE C<sub>8</sub> acid/kg dose group at the three- and six-month sacrifice period. Tissues were harvested for histopathologic examination and CTFE acid analysis. This study is presently in its ninth month of a scheduled one-year time period.

#### INTRODUCTION

Chlorotrifluoroethylene (halocarbon 3.1 oil) oligomer is a nonflammable, saturated, and hydrogen-free chlorofluorocarbon oil. It is noncorrosive, has high thermal stability, good lubricity, and high dielectric strength. A recently concluded 90-day inhalation study to 1000, 500, and 250 mg CTFE/m<sup>3</sup> in this laboratory resulted in a dose-dependent depression in body weight gains of male rats (Kinkead et al., 1989). Alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase (ALT) values examined at the conclusion of the study indicated a treatment-response effect in the male test rats but not in the female test rats. Relative kidney and liver weights of the male test rats were elevated significantly when compared to the respective control groups. The primary target organ of CTFE was the liver. Gross liver hypertrophy and microscopic hepatocytomegaly were the principal manifestations of CTFE-induced hepatotoxicity. The liver alterations were similar to that seen following acute exposure to perfluorodecanoic acid. This suggests that CTFE toxicity may be due to the formation of acid metabolites. Halocarbon 3.1 oil is an isometric mixture of trimeric and tetrameric oligomers that, when chromatographed, results in two distinct groups of peaks. The contributions of CTFE trimer acid (corresponding to the first set of peaks) and tetramer acid (corresponding to the second set of peaks) to the overall toxicity of CTFE were addressed independently.

This study was designed to determine the maximum tolerated dose level in rats by the repeated oral route of administration. The maximum concentration of acid chosen was equivalent to that determined to be in the liver at the end of the 90-day inhalation study with CTFE oligomers

(0.25 mg/L). This was the level at which no effect was noted on body weight increase (Kinkead et al., 1989). Determinations were made by use of the physiologically based pharmacokinetic (PB-PK) model developed for CTFE.

#### MATERIALS AND METHODS

##### *Animals*

Upon receipt from Charles River Breeding Labs, Kingston, NY, the Fischer 344 (F-344) rats were quality control tested prior to use in the studies. The animals were randomized using a proprietary modular software system (PATH/TOX® System, Xybion Medical Systems, Cedar Knolls, NJ) that assigned animals to groups. The animals were group housed (three per cage) in clear plastic cages with wood-chip bedding. The male rats in the one-year repeated-dose study were housed in metabolic cages for a 24-h period each week (1300 h Monday through 1300 h Tuesday) for the first six months, then every third week during the final six months. Water and feed (Purina Formulab #5008) were available ad libitum except for 12 h prior to sacrifice. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

##### *Test Agent*

The CTFE acid samples used in this study were purchased by the Air Force from Technolube Products, Inc., Ultrasystems, Inc., Irvine, CA. The samples were identified as follows.

##### CTFE Trimer acid

Sample #1	Sample #2
ID#10-86-40 IR#14086	ID#10-86-63 IR#14240
B.P. 82-85C/10-3 mm Hg	Eq. Wt. 357.8 M.W. 363.5
Eq. Wt. 357.1 M.W. 363.5	

##### CTFE Tetramer acid

Sample #1	Sample #2
ID#10-86-44 IR#14132	ID#10-86-59 IR#14209
B.P. 110-124C/10-3 mm Hg	Eq. Wt. 475.6 M.W. 480.0
Eq. Wt. 474.6 M.W. 480	

##### *Treatment Regimen*

The male rats were dosed once weekly over a 12-month period. Dosing occurred each Wednesday; the animals were not fasted prior to dosing. The compounds were prepared in corn oil and were gavaged at a constant volume of 0.01 mL/g body weight. The dose concentrations of the trimer and tetramer acids chosen were equivalent to that determined to be in the liver at the end of the 90-day inhalation study with the parent CTFE oligomers (0.25 mg/L, Kinkead et al., 1989). This was

the level at which no effect was noted on body weight increase. Two lower dose levels (decreased by a multiple of 0.5) also were tested. All animals received an initial dose, calculated to produce a predetermined liver-CTFE concentration, followed by a weekly maintenance dose which was expected to maintain a stable liver-CTFE concentration. The concentrations, determined by using the PB-PK model developed for CTFE, are shown in Table 3.9-1.

TABLE 3.9-1. DOSING REGIMEN FOR REPEATED ORAL DOSING OF F-344 RATS WITH CTFE TRIMER AND TETRAMER ACIDS

Group	Initial Dose (mg/kg)	Maintenance Dose (mg/kg)	Number Male Rats
<b>Trimeric Acid</b>			
I	98	6.62	12
II	49	3.31	12
III	25	1.66	12
<b>Tetrameric Acid</b>			
IV	32	2.16	12
V	16	1.08	12
VI	8	0.54	12
<b>Control - Corn Oil</b>			
VII	0.01 mL/g	0.01 mL/g	12

Body weights were recorded weekly prior to each dose administration. Three animals per group were maintained in metabolic cages for a 24-h period on the above-mentioned schedule at which time urine, fecal, and blood samples were collected. Three rats per group are sacrificed serially every three months during the one-year study. At sacrifice, blood samples were collected for clinical chemistry determinations, gross observations were made, and livers were weighed. Sections of liver were prepared for peroxisomal  $\beta$ -oxidation assays, CTFE acid metabolite analysis, and for examination by light microscopy.

#### *Analyses of Biological Samples*

This is the subject of another report (see report number 3.2).

#### *Liver Enzyme Assay*

Peroxisomal  $\beta$ -oxidation assays were performed on approximately 1 g of liver tissue. Samples were removed from the median lobe and placed in ice-cold 0.25 M sucrose. The sample was homogenized, then centrifuged at 1500  $\times$  g for 10 min. A portion of the resultant supernatant was used to measure the rate of palmitoyl coenzyme A oxidation.

#### *Statistical Analysis*

Comparisons of mean body weights were performed using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A two-factorial analysis of

variance with multivariate comparisons was used to analyze the histopathology, clinical chemistry, and organ weight data. The histopathology data were analyzed using one of the following nonparametric tests: Fisher's Exact Test, or if not valid, Yates' Corrected Chi-square (Zar, 1974). Peroxisomal  $\beta$ -oxidation assays were analyzed using a nonparametric analysis of variance (SAS, 1985). A probability of 0.05 inferred a significant change from controls.

## RESULTS

This study is now in the ninth month of its scheduled one-year duration. A significant depression ( $p < 0.05$ ) in mean body weight occurred in the high-dose tetramer group beginning at Day 8 and continues to date (Figure 3.9-1). The mean body weight of the mid-dose tetramer rats has consistently been lighter than the low-dose and control rats, but the differences have not been statistically significant. All trimer-dosed rat groups are maintaining mean body weights comparable to the control rat group.

Blood chemistry data collected at the first interim (three-month) sacrifice for trimer-dosed rats appeared essentially normal. Alkaline phosphatase values of the 1.08- and 2.16-mg tetramer/kg groups were elevated, with the difference statistically significant ( $p < 0.05$ ) in the high-dose group only. The hematology values obtained at three months from all test groups were within normal hematologic parameters. Similarly, alkaline phosphatase values at the two higher tetramer treatment groups were elevated at six months. Also at six months, ALT values were elevated significantly in the two higher tetramer-treated groups.

Significant increases ( $p < 0.01$ ) in absolute and relative liver weights occurred in the high-dose-level tetramer rats (Table 3.9-2). The relative liver weights of the high-dose group were elevated by greater than 70% compared to the control group. The trimer-dosed rat groups and the low-level, tetramer-dosed groups had relative liver weights that essentially were equivalent to that of the control group.

A significant, dose-related increase in the rate of peroxisomal  $\beta$ -oxidation was noted in the rats receiving weekly oral doses of CTFE tetramer (Table 3.9-3). The rate of peroxisomal  $\beta$ -oxidation measured in the high-dose tetramer group after three months was 5.7 times that of the controls, whereas the mid-dose animals' rate was 2.8 times the rate of the controls. Although the rate measured in the trimer-treated rat groups was greater than the rate of the control group, the difference was statistically significant only at the highest dose level. Enzyme levels examined after six months showed no difference in the trimer-treated rats, and only the highest level tetramer-treated group remained different from controls.

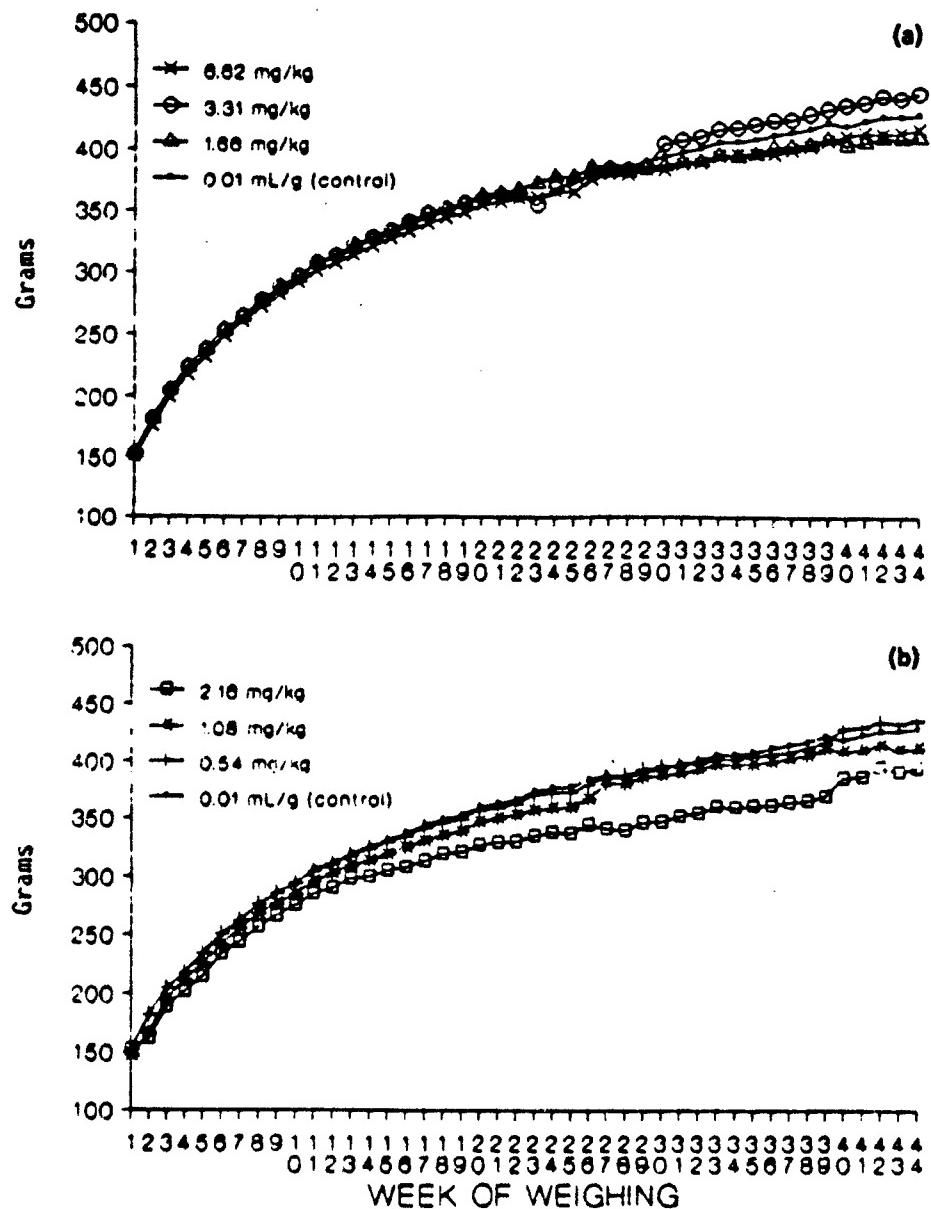


Figure 3.9-1. Effect of Repeated Weekly Oral Dosing of CTFE Trimer Acid (a) and CTFE Tetramer Acid (b) on Body Weight of Male F-344 Rats.

Weekly 24-h urine volumes of the test groups did not differ in volume from that of the control group. The weekly urine volumes from the first 27 weekly samples are listed in Tables 3.9-4 and 3.9-5. This study is continuing and further data will be presented in future annual reports of THRU activities.

TABLE 3.9-2. LIVER/BODY WEIGHT RATIOS (%)<sup>a</sup> OF RATS FOLLOWING WEEKLY ORAL DOSING OF CTFE ACIDS

	Trimer		
	<u>Control</u>	<u>1.66 mg/kg</u>	<u>3.31 mg/kg</u>
3 mo	2.81	2.66	2.65
6 mo	2.58	2.50	2.41
Tetramer			
	<u>Control</u>	<u>0.54 mg/kg</u>	<u>1.08 mg/kg</u>
3 mo	2.81	2.84	3.01
6 mo	2.58	2.59	2.71

<sup>a</sup> Mean  $\pm$  SEM, N = 3 except where indicated.

<sup>b</sup> N = 2.

<sup>c</sup> Significantly different from control, p < 0.01, as determined by a two-factorial analysis of variance with multivariate comparisons.

TABLE 3.9-3. RATE OF PEROXISOMAL OXIDATION<sup>a</sup> IN MALE F-344 RATS FOLLOWING WEEKLY ORAL DOSING OF CTFE ACIDS

CTFE Acid	Control	Low	Medium	High
3 months				
Trimer <sup>b</sup>	2.4 $\pm$ 0.4	3.5 $\pm$ 0.3	4.4 $\pm$ 0.2	5.1 $\pm$ 0.3 <sup>c</sup>
Tetramer <sup>b</sup>	2.4 $\pm$ 0.4	5.0 $\pm$ 0.6 <sup>c</sup>	6.6 $\pm$ 0.7 <sup>c</sup>	13.3 $\pm$ 1.6 <sup>c</sup>
6 months				
Trimer <sup>b</sup>	3.1 $\pm$ 0.5	3.2 $\pm$ 0.2	2.3 $\pm$ 0.6	3.0 $\pm$ 0.3
Tetramer <sup>b</sup>	3.1 $\pm$ 0.5	4.6 $\pm$ 0.6	5.3 $\pm$ 0.5	13.3 $\pm$ 0.7 <sup>c</sup>

<sup>a</sup> Micromoles/minute/gram liver, N = 3.

<sup>b</sup> Dose levels: trimer 6.62, 3.31, 1.66 mg/kg; tetramer 2.16, 1.08, 0.54 mg/kg.

<sup>c</sup> Significantly different than control at p < 0.01 as determined by a Kruskal-Wallis analysis of variance.

**TABLE 3.9-4. URINE OUTPUT (mL/24 h)<sup>a</sup> OF MALE F-344 RATS RECEIVING A WEEKLY ORAL DOSE OF CTFE TRIMER ACID**

Week	Control	1.66 mg/kg	3.31 mg/kg	6.62 mg/kg
0	6.1 ± 0.8	7.3 ± 0.2	7.4 ± 0.3	7.4 ± 0.7
1	7.5 ± 1.7	7.3 ± 0.5	7.8 ± 0.7	7.1 ± 0.7
2	9.5 ± 0.7	9.3 ± 0.7	10.1 ± 0.9	8.0 ± 0.4
3	8.7 ± 2.3	9.7 ± 0.5	10.8 ± 0.5	8.7 ± 0.7
4	8.7 ± 0.7	8.5 ± 0.5	7.3 ± 0.4	7.0 ± 0.2
5	7.4 ± 0.4	8.8 ± 0.5	8.0 ± 0.6	8.0 ± 0.6
6	8.6 ± 0.7	11.1 ± 0.7	10.1 ± 0.2	10.9 ± 0.6
7	7.3 ± 0.1	9.2 ± 0.6	8.0 ± 0.2	7.4 ± 0.8
8	10.1 ± 0.1	10.8 ± 0.6	10.3 ± 0.1	9.7 ± 0.1
9	9.9 ± 0.4	10.7 ± 0.1	10.3 ± 0.5	10.4 ± 0.2
10	8.5 ± 0.4	8.0 ± 0.8	8.6 ± 0.5	7.4 ± 1.0
11	10.4 ± 0.5	11.8 ± 0.4	11.4 ± 0.5	11.5 ± 0.5
12	8.0 ± 0.5	8.9 ± 0.2	9.1 ± 0.1	10.5 ± 0.3
13	9.5 ± 0.9	9.6 ± 0.6	9.4 ± 0.8	9.0 ± 0.4
14	8.2 ± 0.3	9.3 ± 0.7	9.3 ± 0.4	7.8 ± 0.6
15	8.7 ± 0.4	9.4 ± 0.2	9.0 ± 0.6	8.9 ± 0.5
16	7.2 ± 0.9	10.1 ± 0.5	9.7 ± 0.5	9.8 ± 0.9
17	9.1 ± 0.6	9.2 ± 0.2	9.4 ± 0.5	9.6 ± 0.6
18	8.6 ± 0.5	9.8 ± 0.3	10.2 ± 0.7	10.0 ± 0.6
19	8.3 ± 0.5	9.7 ± 0.4	8.4 ± 0.9	8.9 ± 0.6
20	8.2 ± 0.5	8.4 ± 0.2	8.6 ± 0.3	9.2 ± 1.1
21	7.7 ± 0.3	8.1 ± 0.2	8.4 ± 0.2	8.0 ± 0.6
23	7.7 ± 0.9	8.4 ± 0.4	8.2 ± 0.4	6.9 ± 0.7
24	7.5 ± 0.2	8.5 ± 0.6	9.1 ± 0.7	7.6 ± 1.0
27	7.9 ± 0.6	8.1 ± 0.4	7.5 ± 0.7	9.3 ± 1.1

\* Mean ± SEM, N = 3.

TABLE 3.9-5. URINE OUTPUT (mL/24 h)<sup>a</sup> OF MALE F-344 RATS RECEIVING A WEEKLY ORAL DOSE OF CTFE TETRAMER ACID

Week	Control	0.54 mg/kg	1.08 mg/kg	2.16 mg/kg
0	6.1 ± 0.8	7.8 ± 0.6	6.2 ± 0.7	7.7 ± 0.5
1	7.5 ± 1.7	9.4 ± 0.9	6.9 ± 0.8	7.3 ± 0.7
2	9.5 ± 0.7	10.6 ± 0.5	9.2 ± 0.2	9.5 ± 0.6
3	8.7 ± 2.3	11.0 ± 0.6	10.2 ± 0.3	11.1 ± 0.7
4	8.7 ± 0.7	7.9 ± 0.7	8.8 ± 0.6	7.5 ± 0.6
5	7.4 ± 0.4	8.0 ± 0.7	7.8 ± 0.7	7.1 ± 0.0
6	8.6 ± 0.7	10.3 ± 0.5	9.7 ± 0.6	10.2 ± 0.3
7	7.3 ± 0.1	8.4 ± 0.3	7.6 ± 0.4	8.0 ± 0.3
8	10.1 ± 0.1	10.7 ± 0.4	9.6 ± 0.7	10.4 ± 0.2
9	9.9 ± 0.4	10.0 ± 0.6	10.2 ± 0.1	10.8 ± 0.7
10	8.5 ± 0.4	8.3 ± 0.0	7.8 ± 0.3	8.0 ± 0.4
11	10.4 ± 0.5	11.5 ± 0.2	9.7 ± 0.3	11.5 ± 1.0
12	8.0 ± 0.5	8.7 ± 0.2	8.7 ± 0.5	9.8 ± 0.9
13	9.5 ± 0.9	8.9 ± 0.3	8.5 ± 0.4	10.0 ± 0.4
14	8.2 ± 0.3	8.5 ± 0.3	7.9 ± 0.6	8.9 ± 0.7
15	8.7 ± 0.4	9.0 ± 0.4	9.1 ± 0.7	9.6 ± 0.6
16	7.2 ± 0.9	9.0 ± 0.5	8.4 ± 0.1	10.4 ± 0.6
17	9.1 ± 0.6	9.3 ± 0.1	9.4 ± 0.7	10.3 ± 0.2
18	8.6 ± 0.5	9.4 ± 0.0	8.7 ± 0.5	9.6 ± 0.3
19	8.3 ± 0.5	9.0 ± 0.4	8.5 ± 0.2	9.8 ± 0.4
20	8.2 ± 0.5	9.0 ± 0.4	8.3 ± 0.1	8.8 ± 0.4
21	7.7 ± 0.3	8.1 ± 0.4	7.5 ± 0.5	9.1 ± 0.3
23	7.7 ± 0.9	7.7 ± 0.2	8.8 ± 1.0	8.6 ± 0.6
24	7.5 ± 0.2	8.0 ± 0.3	8.0 ± 0.6	9.8 ± 1.1
27	7.9 ± 0.6	9.0 ± 0.9	8.1 ± 0.4	9.7 ± 0.5

\* Mean ± SEM, N = 3.

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### 3.10 EVALUATION OF THE INITIATION/PROMOTION POTENTIAL OF CTFE TRIMER ACID

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#### ABSTRACT

Chlorotrifluoroethylene (CTFE) trimer acid was administered both as a tumor initiator and promoter for three months. Groups of animals were examined after three months of treatment; additional groups of animals will be examined after nine months of treatment. Increases in the liver-to-body weight ratio and liver weight were observed in animals examined after three months, but these increases were the result of phenobarbital administration. There were no increases in the rate of peroxisomal  $\beta$ -oxidation. Quantitation of enzyme-altered foci in livers from these animals has been initiated. A determination of the effects of this agent on relative liver weight,  $\beta$ -oxidation, and development of enzyme-altered foci in animals receiving treatment for nine months has begun.

#### INTRODUCTION

Chronic administration of CTFE oligomers for 90 days by inhalation resulted in hepatomegaly and an increased number of peroxisomes within hepatocytes (Kinkead et al., 1989). A study in which CTFE oligomers were administered by oral gavage for 14 days resulted in similar findings, and, in addition, the rate of cyanide-insensitive peroxisomal  $\beta$ -oxidation of palmitoyl coenzyme A (CoA) was increased (DelRaso, unpublished findings).

Many compounds cause an increase in the number of peroxisomes and a large number of these are structural analogs of the hypolipidemic agent, clofibrate (Lalwani et al., 1983). The proliferative response is not restricted to hypolipidemic agents, however, because numerous industrial chemicals such as phthalate ester plasticizers (Reddy et al., 1976; Moody and Reddy, 1978), agricultural chemicals such as phenoxy acid herbicides (Vainio et al., 1983; Kawashima et al., 1984), and even a high-fat diet (Ishii et al., 1980) also can induce peroxisomal proliferation. Several peroxisome proliferators have been shown to inhibit mitochondrial fatty acid oxidation in rat liver (Bone et al., 1982; Horie and Suga, 1985; Elcombe and Mitchell, 1986; Draye and Vamecq, 1987; Foxworthy and Eacho, 1988; Eacho and Foxworthy, 1988), which has suggested that inhibition of mitochondrial  $\beta$ -oxidation may induce peroxisome proliferation as an adaptive response (Sharma et al., 1988).

Recently, perfluoro-*n*-decanoic acid (PFDA), a compound unrelated to known peroxisome proliferators, was shown to result in hepatomegaly (Olson et al., 1982), peroxisomal proliferation (Van Rafeleghem, 1985) and a 20- to 40-fold increase in fatty acyl-CoA oxidase activity, the rate-limiting enzyme in the fatty acid oxidase system (Harrison et al., 1988). This finding and that of McCarthy (1964), who showed that mammals were able to oxidize *n*-paraffins to the corresponding

fatty acids, led to the hypothesis that CTFE oligomers (perhalogenated alkanes of varying carbon chain length) can be metabolized to perhalogenated fatty acids similar to PFDA.

In rodents, the chronic administration of peroxisome proliferators has been shown to cause an increase in benign and malignant hepatic tumors (Reddy et al., 1980; NTP, 1976, 1982; Hartig et al., 1982). The trend for hepatocarcinogenic potency in rodents has been correlated well with peroxisome proliferative potency (Reddy et al., 1980; Elcombe, 1985). Peroxisome proliferators are not mutagenic in bacterial or mammalian cell mutagenicity assays, and they fail to bind to DNA or induce its repair (Warren et al., 1980; Gupta et al., 1985). However, several peroxisome proliferators have been shown to act as tumor-promoting agents (Reddy and Rao, 1978; Schulte-Hermann et al., 1981; Mochizuki et al., 1982).

The following study was designed to provide information on the ability of the CTFE trimer acid to act either as a tumor initiator or promoter.

## MATERIALS AND METHODS

### *Animals*

Male Sprague-Dawley rats (three weeks old) were purchased from Charles River Laboratories (Kingston, NY). They were quality control tested and found to be in acceptable health. The animals were group housed (four per cage) for one week in plastic cages containing hardwood-chip bedding and given a commercial diet (Purina Formula 5008) and water ad libitum. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

### *Test Material*

CTFE trimer acid was purchased by the Air Force from Technolube Products, Inc., Ultrasystems, Inc., Irvine, CA. Pertinent data are provided below.

#### CTFE Trimer Acid

ID# (Lot No.) 10-86-40 IR# 14086  
B.P. 82-85C/10<sup>3</sup> mm Hg  
Eq. wt. 357.1 M.W. 363.5

All solutions of CTFE trimer acid were prepared in sterile saline as the sodium salt and the pH was adjusted to 7.4.

## **EXPERIMENTAL DESIGN**

### **Initiation Assessment**

A total of seven groups consisting of eight animals per group and one consisting of four animals were used. All animals were subjected to a two-thirds partial hepatectomy procedure (Higgins and Anderson, 1931) using isoflurane anesthesia, except for Group F (Table 3.10-1), which was sham hepatectomized. The sham procedure consisted of a laparotomy only. The surgical procedure was followed 24 h later by a single intraperitoneal dose of diethylnitrosamine (DEN, 10 mg/kg) to Group A. Groups B through F were administered CTFE trimer acid (98 mg/kg) by intraperitoneal (ip) injection. This dose was determined by the physiologically based pharmacokinetic CTFE model to be one which would result in liver concentrations similar to those of animals exposed to a 90-day inhalation study with CTFE oligomers (0.25mg/L). This was the level at which no significant effect was noted on body weight. Two weeks after the end of the initiation period with DEN or CTFE trimer acid (initiation period was 14 days for DEN and 1, 10, 20, or 30 days for CTFE trimer acid), all groups were administered phenobarbital (PB, 500 ppm) in the drinking water for the remainder of the study. Three or four animals from each group were selected at the three-month time period following the beginning of PB administration and treated as outlined below. Animals in Group G were not examined at three months because only five animals remained because of mortality. Group G animals will be examined after nine months of treatment.

**TABLE 3.10-1. EXPERIMENTAL DESIGN OF THE INITIATION PHASE**

Parameter	Group						
	A	B	C	D	E	F	G
pH	+	+	+	+	+	-	+
Initiator	DEN <sup>a</sup>	Acid <sup>b</sup>	-				
# Days <sup>c</sup>	14	1	10	20	30	30	-
Promoter	PB <sup>d</sup>	PB <sup>d</sup>	PB <sup>d</sup>	PB <sup>d</sup>	PB <sup>d</sup>	PB <sup>d</sup>	PB <sup>d</sup>

pH = Partial hepatectomy

DEN = Diethylnitrosamine

Acid = CTFE trimer acid

PB = Phenobarbital

<sup>a</sup> DEN single ip dose, 10mg/kg in saline

<sup>b</sup> Trimer acid single ip dose, 98 mg/kg

<sup>c</sup> Number of days refers to the length of time between injection of either DEN or trimer acid and the beginning of PB administration.

<sup>d</sup> PB in drinking water (500 ppm)

### **Promotion Assessment**

There were seven groups consisting of eight animals per group in this portion of the study. The experimental animals were subjected to a two-thirds partial hepatectomy with isoflurane anesthesia, except for those in Group Q which were sham-hepatectomized. Twenty-four hours after

hepatectomy, all animals received DEN (10 mg/kg) via ip injection except for Groups Q and R, which received saline. Two weeks after these injections, PB (500 ppm in drinking water) was administered to Group M while CTFE trimer acid was given to Groups N through Q at the doses and frequency shown in Table 3.10-2. Groups R and S received saline injections. Three or four animals from each group were selected at the three-month timepoint following the beginning of administration of either PB or CTFE trimer acid and treated as described in Table 3.10-2.

TABLE 3.10-2. EXPERIMENTAL DESIGN OF THE PROMOTION PHASE

Parameter	Group						
	M	N	O	P	Q	R	S
pH	+	+	+	+	-	+	+
Initiator	DEN <sup>a</sup>	DEN <sup>a</sup>	DEN <sup>a</sup>	DEN <sup>a</sup>	-	-	DEN <sup>a</sup>
Promoter	PBD	Acid <sup>c</sup>	Acid <sup>d</sup>	Acide	Acid <sup>c</sup>	-	-

<sup>a</sup> DEN single ip dose, 10 mg/kg in saline.

<sup>b</sup> PB in drinking water (500 ppm).

<sup>c</sup> Trimer acid (initial dose = 98 mg/kg, maintenance dose = 12.25 mg/kg every two weeks).

<sup>d</sup> Trimer acid (initial dose = 9.8 mg/kg, maintenance dose = 1.23 mg/kg every two weeks).

\* Trimer acid (initial dose = 0.98 mg/kg, maintenance dose = 0.12 mg/kg every two weeks).

After three months the animals were euthanized by CO<sub>2</sub> asphyxiation. The terminal animal weight and the liver weight were obtained for each animal.

#### *Histological and Histochemical Studies*

A cross section from the right anterior lobe was removed and placed in buffered neutral formalin. Following fixation each piece of liver was embedded in paraffin and six serial sections (5-μm thick) were prepared from three separate areas within each paraffin block and stained as follows. The first section from each of the three areas was stained with hematoxylin and eosin. The second section from each area was stained for the presence of iron as described by Hirota and Williams (1979). A third section was stained for the presence of glycogen using the periodic acid/Schiff reaction described by Bedi and Horobin (1976).

A separate piece of liver from the right anterior and posterior lobes was frozen and six serial frozen sections (10-μm thick) were prepared from three separate areas within each piece of liver. Adjacent sections from three different areas within each of the blocks of tissue were stained according to the procedures listed below. One section was stained for the presence of gamma-glutamyltranspeptidase activity using the method described by Rutenburg et al. (1969). A separate adjacent section was stained for the presence of adenosine triphosphatase activity according to the method described by Wachstein and Meisel (1957). The third adjacent area from each of the three areas was stained for the presence of glucose-6-phosphatase activity by the method described by Wachstein and Meisel (1958).

### **Enzyme Studies**

The cyanide-insensitive peroxisomal  $\beta$ -oxidation of palmitoyl CoA procedure of Lazarow (1982) was performed on a 1500  $\times g$  supernatant fraction of a 20% liver homogenate prepared in 0.25 M sucrose. The initial rate of oxidation was expressed as the amount of nicotinamide adenine dinucleotide formed per minute and normalized either to gram of liver or total liver weight.

### **Statistics**

The Kruskal-Wallis analysis of variance test was used to compare body weights, liver-to-body weight ratios, and enzyme data. This data was analyzed further by the Scheffe multiple comparison test.

## **RESULTS**

### **Body and Liver Weight**

There were no significant differences in the terminal animal body weights after three months of treatment. However, there were significant differences in the liver weights of some groups of animals (Table 3.10-3) and these differences were found when the mean liver weights of those groups of animals receiving PB (Groups A through F and M) were compared to the mean liver weights from those not receiving PB. However, the average of the liver-to-body weight ratios from those animals in Groups A through F and M were not significantly different from the average of the liver-to-body weight ratios of the animals in Groups N through S (Table 3.10-4). The liver-to-body weight ratios of animals in Groups N through P (those receiving CTFE trimer acid) were not significantly different from those of controls (Groups Q through S).

**TABLE 3.10-3. LIVER WEIGHT<sup>a</sup> OF MALE F-344 RATS FOLLOWING THREE MONTHS OF PROMOTION WITH EITHER PHENOBARBITAL OR CTFE TRIMER ACID**

Initiation Phase		Promotion Phase	
Group	Weight (g)	Group	Weight (g)
A	29.18 $\pm$ 3.03 <sup>b</sup>	M	31.51 $\pm$ 2.02 <sup>b</sup>
B	24.03 $\pm$ 1.69 <sup>b</sup>	N	16.46 $\pm$ 0.74
C	22.71 $\pm$ 3.98 <sup>b</sup>	O	18.27 $\pm$ 0.75
D	26.34 $\pm$ 1.59 <sup>b</sup>	P	19.36 $\pm$ 0.59
E	22.39 $\pm$ 0.80 <sup>b</sup>	Q	20.48 $\pm$ 0.69
F	30.83 $\pm$ 3.00 <sup>b</sup>	R	18.19 $\pm$ 2.16
		S	19.81 $\pm$ 1.93

<sup>a</sup> Mean  $\pm$  SEM, N = 3 for all groups except groups E, F, O, P, and R where N = 4

<sup>b</sup> The average of all the groups is significantly higher than the average of Groups N through S ( $p < 0.01$ , Scheffe multiple comparison test)

TABLE 3.10-4. LIVER-TO-BODY WEIGHT RATIO<sup>a,b</sup> (%) OF MALE F-344 RATS FOLLOWING THREE MONTHS OF PROMOTION WITH EITHER PHENOBARBITAL OR CTFE TRIMER ACID

Initiation Phase		Promotion Phase	
Group	Ratio (%)	Group	Ratio (%)
A	5.79 ± 0.23	M	5.31 ± 0.43
B	4.70 ± 0.11	N	3.49 ± 0.06
C	4.57 ± 0.50	O	3.68 ± 0.06
D	4.82 ± 0.16	P	3.60 ± 0.02
E	4.80 ± 0.20	Q	3.58 ± 0.05
F	5.37 ± 0.38	R	3.72 ± 0.15
		S	3.67 ± 0.14

<sup>a</sup> Liver weight/body weight × 100

<sup>b</sup> Mean ± SEM, N = 3 for all groups except groups E, F, O, P, and R where N = 4

#### Enzyme Data

There were no significant differences in the activity of cyanide-insensitive palmitoyl CoA oxidation when the initial rate was normalized to gram of liver. However, when these data were normalized to total liver weight, some differences were noted but were found to be due mainly to increases in liver weights as a function of the PB treatment.

#### Enzyme-Altered Foci

All slides have been stained for the presence of foci. An examination of the slides has not revealed any qualitative differences between Groups A and M (positive controls) and the groups receiving CTFE trimer acid either as an initiator or promoter. Quantitation of the foci has been initiated.

#### DISCUSSION

Administration of halocarbon 3.1 oil (a mixture of C6 and C8 CTFE oligomers) resulted in both peroxisomal proliferation and increased activity of peroxisomal β-oxidation of fatty acids (Kinkead et al., 1989). Peroxisomal proliferators have been shown to inhibit mitochondrial fatty acid oxidation in rat liver (Bone et al., 1982; Horie and Suga, 1985; Elcombe and Mitchell, 1986; Droye and Vamecq, 1987; Foxworthy and Eache, 1988; Eacho and Foxworthy, 1988), and it has been proposed that this is the mechanism responsible for the induction of peroxisome proliferation (Sharma, 1988). It has been suggested that the mechanism of mitochondrial inhibition involves the formation of a metabolically inert CoA ester derivative of the peroxisome proliferators, which contain carboxylic acid functional groups (Bronfman et al., 1986). The perhalogenated fatty acid, PFDA, has been shown to be a potent peroxisome proliferator, and it has been proposed that this compound forms metabolically inert CoA esters as well.

The biotransformation of alkanes to fatty acids has been characterized well and mechanisms can be written for the biotransformation of CTFE oligomers to perhalogenated fatty acids, which are similar in structure to PFDA. In a recent study by Kinkead et al. (see Report #3.9) the chemically synthesized fatty acid derivative of C8 CTFE oligomer (tetramer acid) was found to cause a four to sixfold induction of peroxisomal enzymes involved in peroxisomal  $\beta$ -oxidation. The synthesized fatty acid derivative of C6 CTFE oligomer (trimer acid) caused a slight increase after three months of continued dosing but no differences from control were noted following an additional three months of dosing.

The present study was designed to examine the initiation/promotion potential of CTFE trimer acid, the putative six-carbon perhalogenated metabolite of CTFE. Many peroxisome proliferators are hepatocarcinogens and this has been substantiated by numerous studies (NTP, 1976; Reddy and Rao, 1977; Reddy and Qureshi, 1979; Reddy et al., 1979; Reddy et al., 1980; Reddy et al., 1982; Rao et al., 1984). In these studies tumors have been observed only following the chronic administration of doses causing significant induction (several hundred percent) of peroxisomal enzymes involved in fatty acid metabolism. In the absence of a significant degree of enzyme induction, compounds such as clofibrate and di(2-ethylhexyl)phthalate lacked tumorigenic activity (Hartig et al., 1982; NTP, 1982).

Although the studies described above involved the administration of the test agent for one to two years, shorter term initiation/promotion assays of some of these compounds have been performed (Numoto et al., 1984; Perera and Shinozuka, 1984; Popp et al., 1985). In a recent study, Cattley and Popp (1989) found that the highly carcinogenic peroxisome proliferator WY-14,643 was an efficient promoter of hepatocarcinogenesis and that administration of this compound for 22 weeks caused a tenfold increase in the activity of cyanide-insensitive  $\beta$ -oxidation.

After three months of dosing with CTFE trimer acid in the present study, no significant increases in liver weights or liver-to-body weight ratios were observed. The only increases in liver weights were due to administration of PB, which is a known liver mitogen. Increases in the activity of cyanide-insensitive peroxisomal  $\beta$ -oxidation of palmitoyl CoA expressed as micromoles per minute per total liver also were found in those groups receiving PB. When the activity of the  $\beta$ -oxidation was expressed as micromoles per minute per gram of liver, no significant differences were seen. Expression of the value of peroxisomal  $\beta$ -oxidation in terms of per gram of liver affords a more accurate comparison between groups of animals, because any effects of treatment on liver size are not considered in the calculation of enzyme activity.

Staining for the presence of enzyme-altered foci has been accomplished. Slides have been qualitatively examined for the presence of foci. Only those animals receiving DEN and PB (Groups A and M) have shown evidence of foci. Quantitation and stereological analyses of foci present after

three months of treatment have been initiated and a final sacrifice has been scheduled to occur following nine months of treatment.

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### **3.11 ANALYSIS OF METABOLITES OF CTFE AMENABLE TO TRANSESTERIFICATION**

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#### **ABSTRACT**

A hypothesis, based on studies of perhalogenated compounds similar to chlorotrifluoroethylene (CTFE), was proposed to explain the hepatotoxicity associated with exposure by inhalation of rats to the mixed-oligomeric material. The hypothesis states that free carboxylic acids of the perhalogenated material are formed by reductive dehalogenation followed by enzymatic oxidative processes to form biological esters of the metabolite free acids. An assay was developed to transesterify any biological esters formed from CTFE and to convert these esters to the methyl ester in a one-step reaction using a methanolic hydrochloric acid reagent. The analysis of these materials showed that they were present in both the liver tissue and urine in levels amenable to analysis. Ester peaks related to the tetrameric material were relatively more concentrated in the liver tissue extracts, whereas the ester peaks related to the trimeric materials were relatively more concentrated in the urine. The findings are indicative of a process in which acids are formed biologically through one or more metabolic processes. Analysis of methyl esters may well be a valid indicator of the type and extent of biological metabolism of the CTFE material. Recent studies conducted to refine the transesterification methodology indicate that improved extraction of samples and optimized derivatization conditions will result in an increase in recovery for the analytes of interest.

#### **INTRODUCTION**

A hypothesis proposed for the formation of metabolites of CTFE materials is that free carboxylic acids are formed in the liver which are then converted to biological esters by enzymatic reactions (M. Andersen, 1989, personal communication). These studies were designed to determine the concentrations of materials present in tissue and urine samples that are amenable to forming methyl esters using a one-step transesterification process. This process replaced the ester portion of the biological ester with a methyl group. The resulting methyl esters then were analyzed by gas chromatography. Methods were adapted from existing methods used to determine the methyl esters of perfluorodecanoic acid. Esterified materials were extracted from tissue and urine samples for analysis. This methodology has the advantage of providing esters amenable to gas chromatography, whereas biological esters would not be volatile enough to be analyzed by this technique. The major disadvantage of this methodology is that no information on the biological portion of the ester is available. Studies were conducted on several sets of animals exposed to CTFE by inhalation at levels of 250, 50, and 10 mg/m<sup>3</sup> over 90 days. Additional samples were taken from animals exposed one day to 250 and 10 mg/m<sup>3</sup>. Animals from these studies were sacrificed on schedules that permitted

collection of urine and feces over extended time periods and allowed for liver samples to be taken over a time course for analysis.

#### **EXPOSURE AND SACRIFICE OF ANIMALS**

One group was designated as target organ animals and was exposed to 250, 50, and 10 mg CTFE oligomers/m<sup>3</sup>, respectively, by inhalation for 85 days. A second set of animals designated as physiologically based pharmacokinetic (PB-PK) study animals was exposed to 250 and 10 mg CTFE/m<sup>3</sup> for 84 days. At the conclusion of the repeat exposures, a third set of animals was exposed to CTFE oligomers by inhalation for one 6-h period at levels of 250 and 10 mg/m<sup>3</sup>.

Liver and urine samples collected for this study were taken from both the repeat exposure PB-PK animals and the animals exposed for one day to CTFE. Sample collection and sacrifice of animals were staggered to permit a time-course analysis of liver and urine samples.

Repeat-exposure sets of animals were sacrificed in groups of four animals with a set of four control animals sacrificed with each time point. The first sacrifice occurred in the middle of the repeat exposure at 45 days. A series of postexposure sacrifices after the completion of the exposure on Day 85 were accomplished as shown in Table 3.11-1. The sacrifice schedule for the single-exposure animals is given in Table 3.11-2.

**TABLE 3.11-1. SACRIFICE SCHEDULE FOR REPEAT-EXPOSURE PB-PK ANIMALS**

Days Postexposure	# of Animals Sacrificed/Set	250 mg/m <sup>3</sup>	10 mg/m <sup>3</sup>
0 <sup>a</sup>	4	Yes	Yes
2	4	Yes	Yes
4	4	Yes	No
7	4	Yes	No
14	4	Yes	Yes
28	4	Yes	No
63	4	Yes	No
126	4	Yes	No
252	4	Yes	No

<sup>a</sup> 0 days means sacrifice occurred on the day on which the animals were removed from the chambers at the conclusion of the 85-day inhalation exposure. A group of four control animals were sacrificed with each daily sacrifice set.

TABLE 3.11-2. SACRIFICE SCHEDULE FOR SINGLE-EXPOSURE PB-PK ANIMALS

Days Postexposure	# of Animals Sacrificed/Set	250 mg/m <sup>3</sup>
0 <sup>a</sup>	4	Yes
1	4	Yes
2	4	Yes
7	4	Yes
14	4	Yes

\* 0 days means the animals were sacrificed on the day that they were removed from the exposure chambers after a single exposure.

#### SAMPLE COLLECTION

##### *Urine, Blood, and Fecal Samples*

Samples of urine and feces were collected from the animals held in metabolism cages for the first 14 days of postexposure for both the repeat- and single-exposure animals. These samples were analyzed for both parent CTFE and CTFE acid metabolites.

##### *Liver Tissue Samples*

Liver tissue samples were collected from all animals from both sets of exposures at the time of sacrifice and analyzed for both parent CTFE and CTFE acid metabolites.

#### MATERIALS

CTFE trimer and tetramer methyl esters were synthesized by Ultrasystems Laboratories, Irvine, CA, under contract to the Air Force and were used as received. The acetonitrile and hexane used were high performance liquid chromatography (HPLC) grade from Fisher Scientific; the methanolic HCl was from Supelco, Inc., Bellefonte, PA.

#### METHODS

Standards of CTFE trimer and tetramer acid esters were prepared by weighing approximately 1 mg of the ester materials into scintillation vials and dissolving the materials in 10 mL of HPLC-grade n-hexane to prepare a 100-ppm stock standard solution. Aliquots of this stock standard were diluted to produce working standards over the analytical range of interest.

Tissue samples were prepared by homogenizing 1 g of tissue in 10 mL of acetonitrile for 2 min. The resulting mixture then was centrifuged for 20 min at 3000 rpm (approximately 2000 × g). One hundred microliters of the acetonitrile solution was pipetted into a 1.5-mL vial. Two hundred microliters of 3 N methanolic HCl was added, followed by 1 mL of hexane. The vials were tightly capped and placed on a Haake-Buchler mixer/heater. Samples were incubated at 85 °C for 16 h, then

removed and cooled to room temperature. A 1- $\mu$ L aliquot of the hexane layer was carefully removed for analysis.

Urine samples were prepared by pipetting 100  $\mu$ L of urine into a 1.5-mL vial and adding 200  $\mu$ L of 3 N methanolic HCl and 1.0 mL of hexane. The vials were sealed and heated for 16 h at 85 °C. The samples were cooled to room temperature, then centrifuged for 20 min at 3000 rpm. One microliter of the hexane layer was withdrawn for analysis. For samples using an automated injector, the hexane layer was decanted carefully into an injection vial and a 1- $\mu$ L sample of material was injected automatically onto the gas chromatograph (GC). The data obtained from automated samples were partially processed using a Nelson chromatography data system and were entered into the VAX computer directly.

Conditions for GC analysis were as follows.

Column:	Supelcowax-10 30 m x 0.32 mm i.d. capillary
Injector temperature:	200 °C
Detector:	$^{63}\text{Ni}$ Nickel Electron Capture (ECD)
Detector temperature:	300 °C
Injection volume:	1 $\mu$ L using either a Varian or Hewlett-Packard autosampler
Temperature program:	60 °C for 2 min, then programmed at a 10 °C rise/min to 120 °C and held for 10 min. The column then was heated to 200 °C at a 10 °C rise/min and held for 20 min.

Due to the extreme sensitivity of the detectors, samples and standards were analyzed from lowest concentration to highest concentration whenever possible and frequent solvent blanks were interspersed with the sample sets to minimize possible carry-over effects from injection to injection.

Data processing was accomplished by manually entering the concentration-response data for standards into the RS/1 program on the VAX computer. The equation of best fit for the range of standards was calculated and data from each set of analyses were compared to the appropriate set of standards. Electron capture detectors are not inherently linear over wide concentration ranges and the best-fit curves were polynomials.

#### RESULTS AND DISCUSSION

The extraction and analysis scheme used to derivatize and analyze the methyl esters of the proposed metabolites amenable to transesterification is shown in Figure 3.11-1. Typical chromatograms obtained using the conditions described in the Methods and Materials Section for CTFE parent material and the CTFE metabolite methyl esters are shown in Figures 3.11-2 and 3.11-3. Parent CTFE material did not produce ester peaks when treated in a manner identical to the esterifiable material.

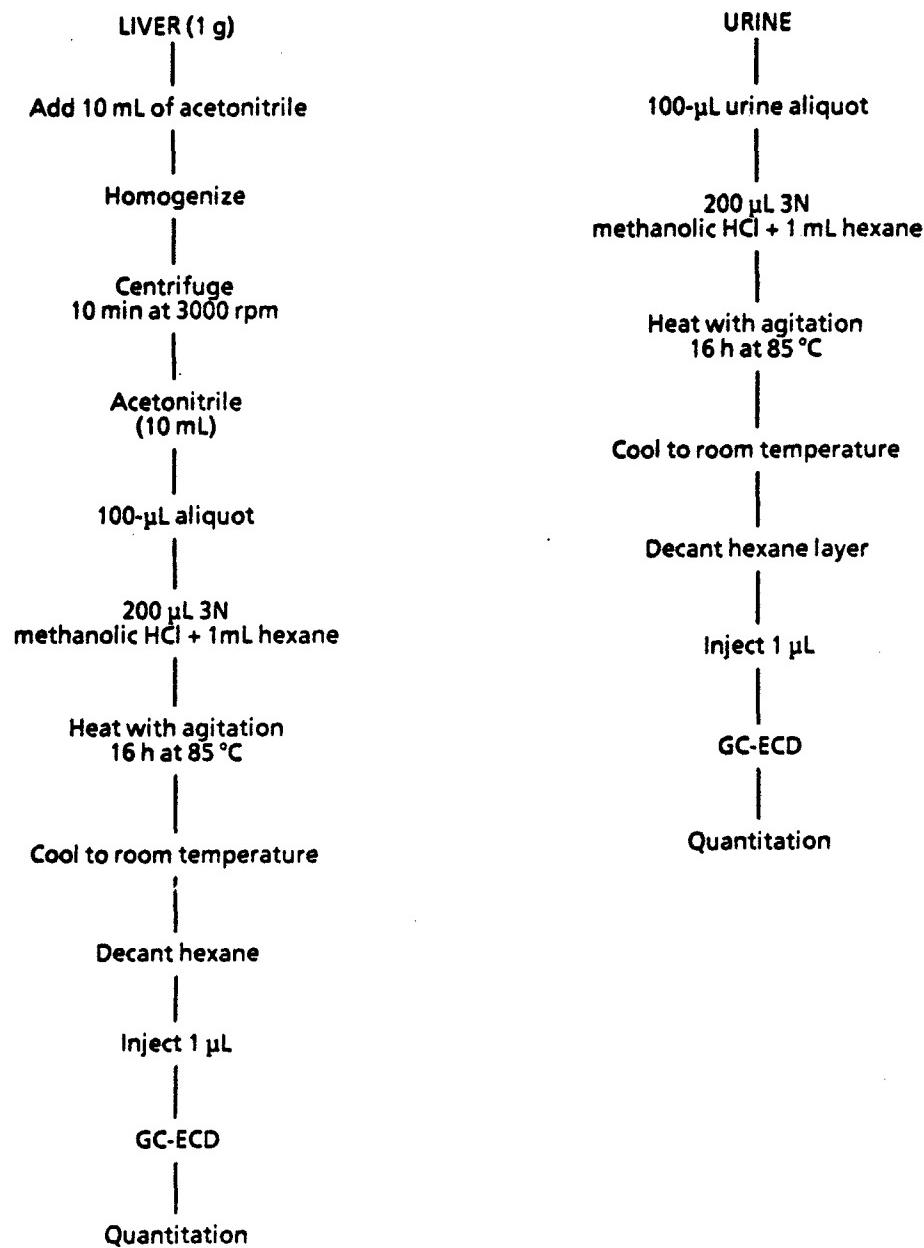


Figure 3.11-1. Analytical Scheme for Methyl Esters of Metabolites.

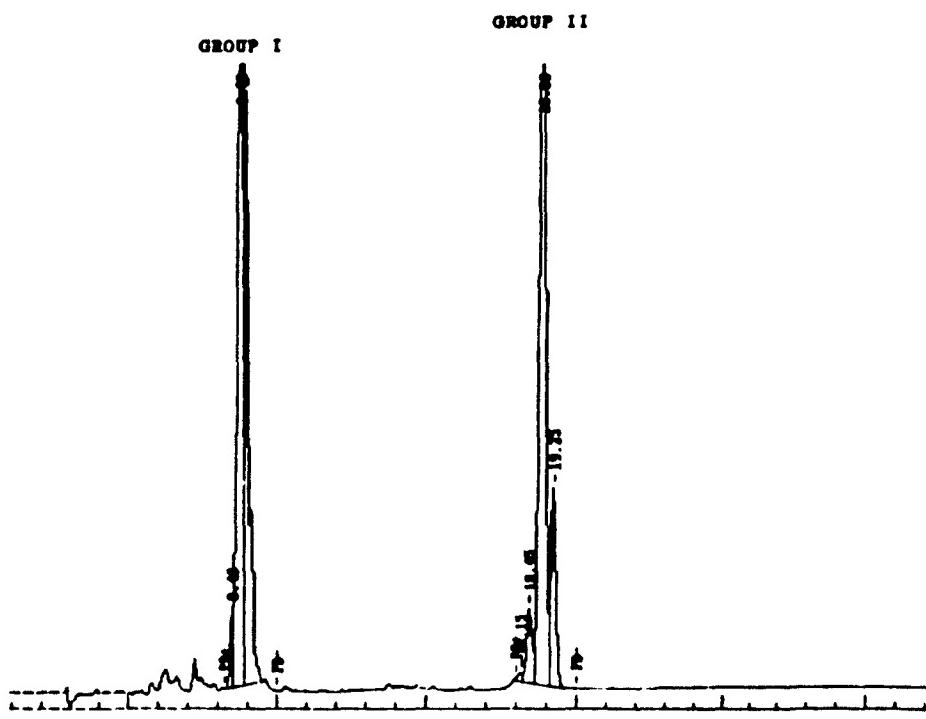


Figure 3.11-2. GC-ECD Chromatogram of CTFE Oligomers.

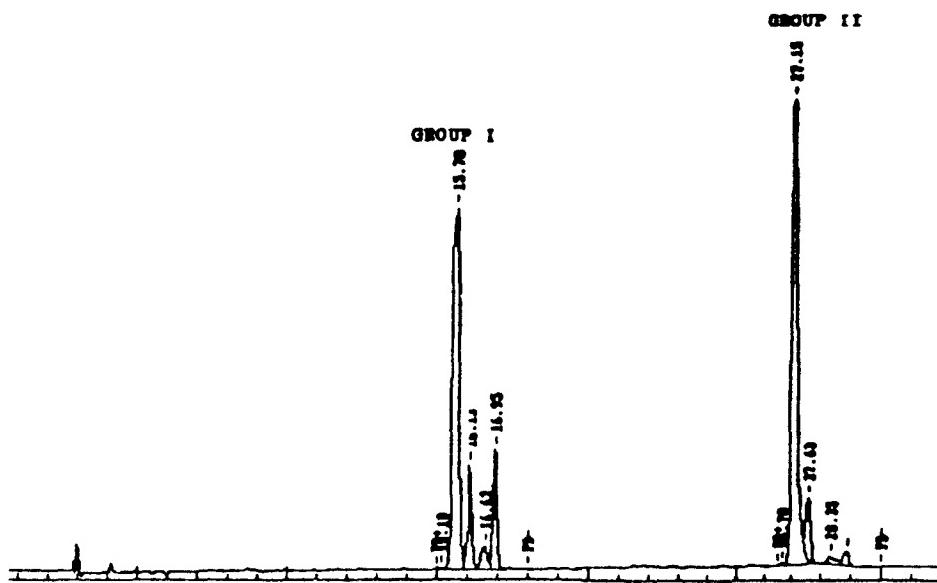


Figure 3.11-3. GC-ECD Chromatogram of Methyl Esters of CTFE Acids.

Gas chromatography of the parent and ester materials divided the CTFE oligomers and methyl esters into two discrete groups, identified in the parent materials as predominantly C6 oligomers (Group I) for the early group of peaks and predominantly C8 oligomers (Group II) for the later eluting peaks. The starting exposure material contained roughly equal amounts of the two groups. These groupings were used to identify the sets of peaks for both parent materials and methyl esters. The significance of the grouping of these materials is illustrated in the tabular data. The early eluting group of peaks for both CTFE and the CTFE acid methyl esters were disproportionately a greater percentage of the total CTFE oligomers in the urine than in the original starting material. Esterifiable material in the urine was several orders of magnitude greater than in the parent CTFE. The late-eluting set of peaks for both parent material and CTFE acid methyl esters were disproportionately a greater percentage of the CTFE oligomers in the liver extracts than in the original starting materials. Tabular data are presented as levels of each group for comparison.

Average postexposure values for CTFE and CTFE acid methyl esters in liver tissue for animals exposed to CTFE by inhalation for 84 or 85 days are shown in Table 3.11-3. Four animals per group were sacrificed at the indicated times. Liver tissues were analyzed for both parent CTFE and CTFE acid methyl esters. The average values for the set of four animals are reported in micrograms per gram of liver tissue.

TABLE 3.11-3. COMPARISON OF LIVER CTFE AND LIVER CTFE METABOLITE VALUES<sup>a</sup> OF ANIMALS EXPOSED 84 OR 85 DAYS TO 250 mg CTFE/m<sup>3</sup>

Days Postexposure	CTFE Parent Concentration		CTFE Metabolite Concentration	
	C6 (Mean)	C8 (Mean)	C6 (Mean)	C8 (Mean)
0	20	54	2	73
2	5	21	5	54
4	3	14	4	59
7	2	12	9	103
14	1	4	4	62
28	0.1	1.5	2	87
63	0.1	1.2	2	21

<sup>a</sup> Micrograms per gram of liver tissue. Control values for CTFE and CTFE metabolites were less than 1.

Note that the levels of C8 oligomers were higher in both sets and that the acid methyl ester peaks continued to show significant levels after 63 days. The metabolite C8 showed an increase in concentration through seven days, whereas the parent material decreased consistently with time. The concentration of the metabolites was more persistent than the corresponding parent material.

Concentrations of CTFE parent material and CTFE methyl ester metabolites in liver tissue for animals exposed to 250 mg CTFE/m<sup>3</sup> by inhalation for one 6-h period are shown in Table 3.11-4. Again, the values presented represent sets of four animals per group sacrificed at the indicated days postexposure.

TABLE 3.11-4. COMPARISON OF LIVER CTFE AND LIVER CTFE METABOLITE VALUES<sup>a</sup> OF ANIMALS EXPOSED FOR ONE SIX-HOUR PERIOD TO 250 mg CTFE/m<sup>3</sup>

Days Post exposure	CTFE Parent Concentration		CTFE Metabolite Concentration	
	C6 (Mean)	C8 (Mean)	C6 (Mean)	C8 (Mean)
0	13.62	9.25	2.11	0.98
1	0.92	2.35	2.42	2.01
2	0.50	0.58	3.33	4.88
7	0.43	0.32	3.59	3.84
14	0.06	0.02	0.63	2.31

<sup>a</sup> Micrograms per gram of liver tissue. Control values for the CTFE parent were below detection limit. Control values for the CTFE metabolites were below 1 µg/g.

The CTFE acid methyl esters increased relative to the parent CTFE materials over time. The increase in retention of the C8 oligomers was not apparent for this short time course, although the parent CTFE material depletes from the liver tissue more rapidly than does the metabolic material.

Urine samples were collected from the same sets of animals sacrificed to obtain liver tissue in the sequential sacrifice studies. Again, analyses were performed for both parent CTFE material and acid metabolites amenable to transesterification by acid hydrolysis. Levels of esterifiable material in the urine were significantly higher than levels of the parent CTFE materials. Single-exposure animals were exposed to 250 mg CTFE/m<sup>3</sup> for one 6-h period. Repeat-exposure animals were exposed for 84 or 85 days. Samples were collected for 14 days postexposure from each exposure group for analysis.

Daily levels of parent CTFE oligomers and of the CTFE acid metabolites found in urine samples of animals exposed to 250 mg CTFE/m<sup>3</sup> by inhalation are reported for 14 days postexposure in Table 3.11-5. Values reported are total micrograms per urine sample and are corrected for percent recovery. The concentration of C6 oligomeric materials was significantly higher in the urine than were the C8 oligomers. This was the opposite of the phenomena observed in the liver where the C8 oligomers were much higher in relative concentration. The original starting material contained roughly equal amounts of the groups of oligomers.

TABLE 3.11-5. COMPARISON OF URINE CTFE AND URINE CTFE METABOLITE VALUES<sup>a</sup> OF ANIMALS EXPOSED 84 OR 85 DAYS TO 250 mg CTFE/m<sup>3</sup>

Days of Sample <sup>b</sup>	CTFE Parent Concentration		CTFE Metabolite Concentration	
	C6 (Mean)	C8 (Mean)	C6 (Mean)	C8 (Mean)
1	0.453	0.138	152	24
2	0.570	0.097	276	38
3	0.509	0.134	302	56
4	0.391	0.090	283	78
5	0.303	0.070	263	68
6	0.322	0.028	213	57
7	0.265	0.040	179	35
8	0.186	0.047	180	38
9	0.205	0.021	203	60
10	0.178	0.011	209	62
11	0.132	0	127	33
12	0.151	0	165	43
13	0.131	0	147	34
14	0.112	0	163	36

<sup>a</sup> Total micrograms per sample.

<sup>b</sup> Sample collected for 24 h after animals were removed from chamber.

Daily levels of parent CTFE and of CTFE metabolites in urine for sets of animals dosed by a single exposure for 6 h to 250 mg CTFE/m<sup>3</sup> by inhalation for 14 days postexposure are shown in Table 3.11-6. In this set of analyses, the early-eluting group of both the parent CTFE and the CTFE acid methyl esters were again excreted into the urine at much higher levels than were the later-eluting materials. The esterifiable material levels were significantly higher than levels of parent CTFE. The absolute amounts of acid ester materials remained fairly constant over the two-week period after reaching a peak concentration on Day 3. The parent CTFE material levels dropped off rapidly after the first day and seemed to reach a constant level during the second week.

#### CONCLUSIONS

Data indicate that esterifiable material is produced by metabolism of CTFE oligomers and that at least a portion of the metabolic material produced is amenable to transesterification using methanolic HCl acid. Ester moieties were more persistent in the body than the parent CTFE oligomers. Distribution of these materials was different in the liver and urine samples analyzed. The original mixture of parent CTFE oligomers used in the inhalation study was about equally distributed between two groups of peaks eluting from the GC under conditions similar to those used in the metabolite

study. The first group contained peaks that were predominantly trimers of the CTFE material end capped with chlorine. The second group of peaks contained mostly tetramers of the CTFE material end capped with chlorine. The data indicate that a disproportionate amount of tetrameric material was persistently stored in the liver whereas the trimeric metabolic material was preferentially excreted in the urine. There were significantly higher levels of esterifiable material present in the urine than the CTFE parent compounds. The efficacy of the method as an indicator of metabolic activity indicates that some form of reductive dehalogenation followed by enzymatic formation of acid methyl esters is a major metabolic process in the rat. Detectable levels of the metabolic esters are observed for a greater time than are the parent CTFE materials, which indicates that these metabolic ester materials may play a significant role in the etiology of the observed hepatotoxicity in rats exposed to CTFE via inhalation.

TABLE 3.11-6. COMPARISON OF URINE CTFE AND URINE CTFE METABOLITE VALUES<sup>a</sup> OF ANIMALS EXPOSED FOR SIX HOURS TO 250 mg CTFE/m<sup>3</sup>

Days of Sample <sup>b</sup>	CTFE Parent Concentration		CTFE Metabolite Concentration	
	C6 (Mean)	C8 (Mean)	C6 (Mean)	C8 (Mean)
1	0.221	0.042	17	0.7
2	0.110	0.017	18	0.4
3	0.117	0	40	0.8
4	0.094	0	31	0.5
5	0.050	0	24	0.4
6	0.057	0	18	0.1
7	0.043	0	19	0.3
8	0.041	0	22	0.3
9	0.025	0	16	0.3
10	0.034	0	18	0.4
11	0.053	0	15	0.5
12	0.058	0	14	0.4
13	0.043	0	11	0.3
14	0.048	0	12	0.3

<sup>a</sup> Total micrograms per sample.

<sup>b</sup> Sample collected 24 h after animals were removed from chamber.

### **3.12 ANALYSIS OF URINARY FLUORIDE LEVELS FROM INHALATION AND ORAL DOSING STUDIES OF RATS WITH CTFE AND CTFE ACIDS**

**W.R. Sayers, D.A. Mahle, C.S. Seckel,  
S.K. Bunker, J.L. Wilson, and H.C. Higman**

#### **ABSTRACT**

Several studies were conducted to determine the effects of inhalation exposure to chlorotrifluoroethylene (CTFE) oligomers and oral gavage of CTFE acids using rats. Animals from these various studies were placed in metabolism cages and urine and feces were collected at time points designed to monitor excretion of inorganic fluoride. Urine samples were analyzed using a combination fluoride electrode. The results of these studies indicate elevated levels of urinary fluoride in animals that received high-level doses of parent CTFE. The oral gavage studies using free carboxylic acids synthesized from CTFE oligomers did not show a corresponding elevation in fluoride levels. This indicates that the free acids were not further metabolized by loss of additional fluoride from the molecule. The significant increase in urinary fluoride levels is indicative of metabolism of the parent compounds, possibly by reductive dehalogenation, which would produce elevated levels of inorganic fluoride.

#### **INTRODUCTION:**

Inhalation studies were conducted exposing Fischer (F-344) rats to CTFE oligomers at 250, 50, and 10 mg/m<sup>3</sup> by repeat inhalation exposure for 85 days to determine target organ toxicity. These animals were monitored for urine fluoride content postexposure. A second set of rats was exposed concurrently by repeat inhalation exposure for 84 days at the 250- and 10-mg/m<sup>3</sup> levels for use in pharmacokinetic modeling experiments. A third set of rats was exposed for one 6-h period for use in pharmacokinetic modeling experiments. Animals from these groups were placed in metabolism cages for designated time periods for collection of urine and fecal material.

A series of oral gavage studies using free acids synthesized from CTFE oligomers was initiated to determine LD<sub>50</sub>, LD<sub>10</sub>, and long-term effects. Both male and female F-344 rats were used in these studies at selected dose levels. The rats from the LD<sub>10</sub> and long-term effects study were placed in metabolism cages for collection of urine and fecal material at designated time points for analysis of urine fluoride content. Fecal materials were stored for future analysis of the parent materials.

## METHODS

### Instrumentation

Fluoride measurements were performed using an Orion model 701A equipped with an Orion model 96-09-00 combination fluoride electrode (Neefus et al., 1970).

The instrument was calibrated using standard fluoride solutions prepared from sodium fluoride. Millivolt readings were plotted versus the log of the fluoride concentration to obtain calibration lines over a concentration range. Least squares linear regression lines were then calculated from standards data and were used to calculate fluoride concentration from samples.

### Materials

Reagent-grade chemicals were used to prepare all buffer and standard solutions. Standard stock solutions were prepared by dissolving sodium fluoride (Matheson SX/550CB721) in deionized water. Calibration standards were prepared by serial dilution of the sodium fluoride solution in simulated urine (Table 3.12-1) mixed with equal volumes of total ionic strength adjustment buffer (TISAB) solution (Orion Research, Cambridge, MA).

### Urine Analysis

Urine was collected from animals in metabolism cages. The volume and weight of the collected urine were recorded. Standards were prepared in simulated urine mixed with equal amounts of TISAB solution and were analyzed. The concentration and materials used to prepare simulated urine for use with calibration standards are shown in Table 3.12-1. Urine samples were prepared by mixing the urine with an equal volume of buffer and analyzing the resulting mixture.

TABLE 3.12-1. SIMULATED URINE COMPONENTS

Compound	Amount
Ammonium phosphate, dibasic	2.00 g
Sodium chloride	11.60 g
Sulfuric acid, concentrated	1.00 mL
Deionized water	1.00 L

## RESULTS AND DISCUSSION

### Inhalation Studies

Groups of F-344 rats were exposed by inhalation to mixed CTFE oligomers at levels of 250, 50, and 10 mg/m<sup>3</sup> for 85 days, 6 h/day, five days per week for target organ toxicity testing. Additional sets of animals were exposed under identical conditions for pharmacokinetic modeling studies at the

250- and 10-mg CTFE/m<sup>3</sup> levels. A third set of animals was exposed to the 250-mg CTFE/m<sup>3</sup> level for one 6-h period. Fluoride data were calculated in units of total micrograms excreted per 24-h period after factoring for the weight and volume of urine collected by comparison to standard curves generated daily for each analytical set. The data are presented as percentages of control values to illustrate the differences. Urinary fluoride levels for two weeks postexposure are shown in Table 3.12-2. Each data point in this table represents the average value obtained for four animals in each subset. The standard deviations for these measurements are not shown but range between 5 and 25%.

TABLE 3.12-2. LEVELS<sup>a</sup> OF URINARY FLUORIDE FROM F-344 RATS EXPOSED BY INHALATION TO CTFE AT 1 TO 14 DAYS POSTEXPOSURE

Day Postexposure <sup>b</sup>	Repeat Exposure Sets (mg/m <sup>3</sup> )			Single Exposure Set (mg/m <sup>3</sup> )
	10	50	250	250
1	86		182	164
2	96	138	291	101
3	109	108	271	109
4	86	171	298	140
5	97	119	353	110
6	99	121	300	109
7	100	147	253	90
8	133	123	349	99
9	128	172	375	91
10	76	126	288	94
11	127	170	397	61
12	138	96	397	81
13	89	123	255	134
14	136	129	295	70

<sup>a</sup> Expressed as a percent of the average control values for the individual daily control animal sets.

<sup>b</sup> Sample collected for 24 h after animals were removed from chamber at conclusion of repeat-exposure study.

The data from the analysis of urine fluoride for animals exposed by inhalation show that there is a definite increase in urine fluoride for animals exposed at the 250-mg CTFE/m<sup>3</sup> level repeatedly over 84 or 85 days when monitored for 14 days postexposure. Animals exposed repeatedly to 50 mg CTFE/m<sup>3</sup> showed a slight elevation in urine fluoride, which did not prove to be statistically significant. The 10-mg/m<sup>3</sup> animals were indistinguishable from the control sets. Animals exposed for one 6-h period at the 250-mg/m<sup>3</sup> level showed no significant increase in urine fluoride when monitored for 14 days postexposure.

### **Oral Gavage Studies Using CTFE Free Acids**

A working hypothesis was developed over the course of the overall study that stated that free carboxylic acids of the CTFE oligomers were formed in the liver by reductive dehalogenation to form biologically active species that result in hepatotoxicity (M. Andersen, 1989, personal communication). In order to test this hypothesis, free carboxylic acids of trimer and tetramer CTFE materials were synthesized under contract to the Air Force for use in determining the toxicity of CTFE acids. Sets of F-344 rats were dosed by oral gavage with free carboxylic acid CTFE compounds in two separate studies. All animals in the two studies were dosed with free acids in corn oil. Control animals were dosed with pure corn oil. The first study involved dosing both male and female rats at an effective LD<sub>10</sub> dose level one time and monitoring the urine fluoride for 14 days. The second study involved dosing six discrete sets of animals with three levels each of both trimer and tetramer acids each Wednesday for one year with weekly urine collection taken each Tuesday through 24 weeks. After 24 weeks, the urine samples were taken every third Tuesday. This study is continuing at present. However, there are sufficient data from the one-year study to determine the trends in the data sets. The dosing regimen for the oral gavage study (Table 3.12-3) provided three samples per dose group plus three control animal samples. To date, there are no statistically significant elevations in urine fluoride levels.

**TABLE 3.12-3. DOSE LEVELS<sup>a</sup> OF TRIMER AND TETRAMER ACIDS BY ORAL GAVAGE**

Trimer Acids (mg/kg)	Tetramer Acids (mg/kg)
1.66	0.54
3.31	1.08
6.62	2.16

\* Dosed in corn oil by oral gavage every Wednesday. Study initiated on 31 Jan 89. Weekly samples taken on Tuesdays for fluoride analysis through 18 July 89, and every three weeks thereafter. Sampling to continue through 31 Jan 90.

### **CONCLUSIONS**

Data from the analysis of urinary fluoride has provided information about the possible metabolism of CTFE oligomers and elevated levels of inorganic materials generated above control values for animals dosed by inhalation and oral gavage. There was a statistically significant increase in urinary fluoride levels in the high-level, repeat-inhalation exposure groups in which there is a defined toxic effect from the CTFE oligomers. There has been no elevation in urinary fluoride levels in the rats dosed with free-acid analogues of CTFE tetramer and trimer acids by oral gavage. This is consistent with the prediction of a free-acid intermediate because the free-acid is already stripped of the reactive fluorine. Studies are continuing to define the long-term effects of the free-acid analogues of CTFE in the rat by oral gavage. The significance in positive elevations of urinary fluoride

in animals exhibiting toxic effects in these studies is indicative of metabolic processes capable of removing fluoride from the parent CTFE oligomers.

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### **3.13      EVALUATION OF THE ACUTE TOXICITY OF SILAHYDROCARBON**

**E.R. Kinkead, S.K. Bunger, and R.E. Wolfe**

#### **ABSTRACT**

Silahydrocarbon (SHC) is a base stock for a candidate high-temperature hydraulic fluid. A complete battery of acute toxicity tests was performed to determine the potential health hazard of the fluid. A single neat dose of 0.1 mL SHC into New Zealand white (NZW) rabbit eyes resulted in slight conjunctival irritation 1 h after treatment in all unflushed eyes and one of three flushed eyes. Conjunctival irritation persisted through 24 h but had dissipated by 48 h. Rinsing the eyes after treatment was of questionable benefit. Single treatment of 0.5 mL neat SHC to rabbit skin produced negative results for all but a single animal, which had very slight erythema at the test site 48 h after treatment. Guinea pigs failed to elicit a sensitization response following repeated application of SHC. A single oral dose of 5 g SHC/kg of animal body weight to five male and five female Fischer 344 (F-344) rats, and a single dermal application of 2 g SHC/kg body weight applied to five male and five female NZW rabbits resulted in no deaths. No signs of toxic stress were observed, and all animals gained weight during the 14-day posttreatment observation periods. Inhalation exposure to SHC was performed by exposing five male and five female F-344 rats to a 4.8 mg/L near-limit concentration of aerosolized SHC for 4 h. All surviving rats gained weight during the 14-day posttreatment observation period.

#### **INTRODUCTION**

SHC is a base stock for a candidate high-temperature hydraulic fluid developed and made by the Air Force Materials Laboratory (AFWAL/MLBT). The Materials Laboratory is presently conducting compatibility tests of SHC with high-temperature hydraulic seals and advanced structural materials. At present, the Materials Laboratory has completed a 500-h low-temperature (275 °C) pump test. Because Air Force personnel already are working with SHC and SHC shows promise as a base stock for hydraulic fluids, potential acute toxicity hazards must be addressed.

The most significant exposure routes for hydraulic fluids are expected to be dermal, due to spills or leaks, and aerosol inhalation from pressurized system leaks. This study addressed these potential routes of exposure and included experiments designed to assess eye and skin irritation, skin sensitization, single-dose oral and dermal toxicity, and aerosol inhalation toxicity. Species and sex of animals selected for these acute toxicity tests were in conformance with the Health Effects Testing Guidelines of the Environmental Protection Agency (1985). Existing alternative methods to animal testing were inadequate for use in this study.

## MATERIALS

### Test Agent

The test material used in this study was supplied by the Air Force. In general, SHCs are defined as compounds represented by the structure SiR<sub>4</sub>. The sample tested was identified as follows.

#### Silahydrocarbon MLO 86-348

##### Pertinent physical characteristics

Flash point (°C):	221
Fire point (°C):	237
Density @ 25 °C:	0.8145
Density @ 100 °C:	0.7671

##### Mol. Wt. (principal "R" components)

Methyltriocyl	382.1
Methyl Octyldecyl	410.1
Methyloctyldidecyl	438.1

##### Viscosity (cSt) at

-54 °C (-65 °F)	2168
-40 °C (-40 °F)	548
40 °C (104 °F)	9.20
100 °C (212 °F)	2.71

### Animals

Male F-344 rats weighing between 100 and 125 g and female F-344 rats weighing between 75 and 100 g were purchased from Charles River Breeding Labs, Kingston, NY. Male Hartley guinea pigs weighing between 200 and 250 g were purchased from Murphy Breeding Labs, Plainfield, IN. Male and female NZW rabbits weighing between 2 and 3 kg were purchased from Clerco Research Farms, Cincinnati, OH. All animals were subjected to a two-week quarantine period. Rats were group housed (three per cage) in clear plastic cages with wood-chip bedding. The guinea pigs and rabbits were housed individually, the guinea pigs in plastic cages with wood-chip bedding, and the rabbits in wire-bottom, stainless-steel cages. Water and feed (Purina Rabbit Chow #5320, Purina Formulab #5008 for rats, and Purina Formulab #5025 for guinea pigs) were available ad libitum, except during the inhalation exposure period and for 16 h prior to oral dosing. Animal room temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals.

## EXPERIMENTAL APPROACH

### Eye Irritation

Nine NZW female rabbits, weighing between 2 and 3 kg, were examined with fluorescein stain prior to use to ensure absence of lesions or injury. A topical anesthetic (Ophthethic, Allergan Inc., Irvine, CA; proparacaine HCl 0.5%) was instilled in the eyes of all rabbits, treated and control,

approximately 2 min prior to application of the test material. One tenth of a milliliter of the test material was applied to one eye of each of the nine rabbits. The opposite eye was left untreated and served as the control. The treated eye of three rabbits was flushed with lukewarm deionized water for 1 min starting 30 sec after instillation. The eyes of the remaining six rabbits were not flushed. Examinations for gross signs of eye irritation were made at 1, 24, 48, and 72 h following treatment. Irritation was scored according to the method of Draize et al. (1944), in which the total score for the eye was the sum of all scores obtained for the cornea, iris, and conjunctivae.

#### ***Skin Irritation***

Six NZW female rabbits were clipped on the back and sides 24 h prior to dosing to allow for recovery of the skin from any abrasion resulting from the clipping. The test agent (0.5 mL) was applied to a designated patch area and was covered by a 3-cm square of surgical gauze two single layers thick. Strips of surgical adhesive tape held the gauze patch in place and the entire shaved area was covered with dental dam and secured with Vetrap® (3M Corp., Minneapolis, MN) and adhesive tape. The patches remained in place for 4 h, then all wrappings were removed and the residual test agent was wiped from the skin. Test areas were evaluated for irritation using the Draize table (Draize et al., 1959) as a reference standard at 4, 24, 48, and 72 h. Total scores of the four observations for all rabbits were divided by 24 to yield a primary irritation rating, which was interpreted using the National Institute for Occupational Safety and Health skin test rating (Campbell et al., 1975).

#### ***Sensitization***

Prior to the start of the study, 10 male guinea pigs were treated on the clipped left flank with 0.1 mL of the undiluted test material to determine the baseline irritation response. The site of the sensitization test was an area just behind the shoulder girdle. This site was clipped with an Oster® animal clipper and depilated with a commercial depilatory (Surgex Hair Remover Cream, Sparta Instrument Corp., Hayward, CA) 4 h prior to treatment. A Vetrap® frame with a 1.5- x 1.5-cm opening was affixed to the guinea pig at the site of the depilated area. One-tenth of a milliliter of the test material was topically applied to the test area and covered with gauze, dental dam, and adhesive tape. This was done on Mondays, Wednesdays, and Fridays until a total of four sensitizing treatments were applied and evaluated. At the time of the third sensitizing treatment, 0.2 mL of a 50% aqueous dilution of Freund's adjuvant (Bacto Adjuvant Complete, Freund, Difco Laboratories, Detroit, MI) per animal was injected intradermally using two or three sites next to the test site. Following the fourth sensitizing treatment, the animals were rested for two weeks. Both flanks then were clipped and one flank was challenged with 0.1 mL of the test material. The challenge application was not occluded. The skin response at these sites was recorded at 4, 12, 24, and 48 h after application. Any animal eliciting a score of two or more at the test solution challenge site at the 48-h scoring interval was

rated a positive responder. The percentage of animals responding was the important factor in determining sensitization potential.

#### ***Oral Toxicity***

Five male and five female F-344 rats were fasted 16 h prior to the administration of the oral dose. Each rat was weighed prior to oral-gavage dosing and 5 g/kg of neat compound was administered. Surviving rats were weighed at 1, 2, 4, 7, 10, and 14 days postexposure and signs of toxicity were recorded. On the 14th day postexposure, rats were sacrificed and gross pathology was performed.

#### ***Dermal Toxicity***

Twenty-four hours prior to dosing, the back and sides of five male and five female NZW rabbits were clipped. The undiluted dose of 2 g/kg was applied to the back of the rabbits and spread evenly to both sides. The dose was kept in place by applying an eight-ply gauze patch over the liquid. A clear plastic wrap then was applied over the entire midsection and was held in place with Vetrap® and Elastoplast® tape. The dose was kept in contact with the rabbit skin for 24 h. The tape, plastic wrap, and gauze then were removed and the residual test material was wiped from the animal. Animal body weights were recorded on Days 1, 2, 4, 7, 10, and 14 posttreatment. Signs of toxicity and mortality were monitored and gross pathology was performed at the termination of the study.

#### ***Inhalation Exposure***

The aerosol generation system consisted of a 250-mL, round-bottom flask containing a six-jet Collison (BGI, Inc., Waltham, MA) compressed air nebulizer operated at a pressure of 50 psi. The 690-L chamber was operated at a mean flow rate of 0.79 cfm providing two chamber volumes of air per hour. Exposure concentration was controlled by adjusting the chamber air flow.

Eight Metrcel (Gelman Sciences, Inc., Ann Arbor, MI) membrane filter samples of chamber atmospheres were taken during the course of the 4-h exposure. These were gravimetrically analyzed for mass concentration by dividing the weight change by the volume sampled. Aerosol particle size distributions were measured with a Lovelace multijet impactor (Intox Products, Albuquerque, NM). Two 30-sec samples were taken during the exposure. A Varian 3700 (Varian Associates, Palo Alto, CA) gas chromatograph equipped with a 3-m, 0.25-mm fused silica SE-30 capillary column and flame ionization detector was utilized to obtain profiles of the SHC as received, as aerosolized, and as a residue from the nebulizer system.

Five male and five female F-344 rats were placed in a 690-L chamber and exposed for 4 h to a target 5 mg/L (limit test) concentration of aerosolized test material. Records were maintained for

body weights (Days 0, 7, 10, and 14 postexposure), signs of toxicity, and mortality. At sacrifice, gross pathology was performed and lungs were removed for histopathologic evaluation.

#### ***Statistical Analysis***

Mean body weights of the inhalation study rats were compared using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983). A probability of 0.05 or less inferred a significant change from controls.

### **RESULTS**

#### ***Eye Irritation***

The instillation of 0.1 mL of SHC in rabbit eyes produced no corneal opacity or congestion, swelling, or discharge of the iris when test animals were observed at 1, 24, 48, or 72 h postinstillation. However, slight irritation of the conjunctivae was noted in all unflushed eyes and one of the three flushed eyes 1 h after treatment. Conjunctival irritation persisted through the 24-h readings but had dissipated by 48 h.

#### ***Skin Irritation***

Six rabbits were treated dermally with 0.5 mL of SHC. No erythema, edema, or necrosis was observed in any of the rabbits upon examination immediately following 4-h dermal contact with the test agent. Subsequent observations at 24, 48, and 72 h were also negative, except for a single animal that had very slight erythema at the test site 48 h after treatment.

#### ***Sensitization***

No test animals exhibited erythema or edema following the baseline response treatment of 0.1 mL test material to the shaved flank. Following 10 days of sensitization dosing and two weeks of rest, the test animals were challenged with 0.1 mL of the test material. The SHC produced no erythema or edema at 24 or 48 h after this challenge treatment.

#### ***Oral Toxicity***

Five male and five female rats were orally dosed with 5 g SHC/kg body weight. No deaths resulted from the oral administration of the test agent and no signs of toxicity were observed. All rats gained weight during the 14-day observation period.

#### ***Dermal Toxicity***

Five male and five female rabbits were treated with 2 g SHC/kg body weight. No deaths occurred from the 24-h contact with the test agent and no signs of toxicity were observed. All rabbits gained weight during the 14-day observation period.

### **Inhalation Toxicity**

Eight filter samples were obtained from the chamber during the 4-h exposure. The mean time-weighted chamber concentration was 4.81 mg/L. Mass median aerosol diameter of the sampled test material was 1.58  $\mu\text{m}$  with a standard geometric mean of 1.93. Gas chromatographic analysis of filtered chamber air revealed no SHC in the vapor phase.

Gas chromatographic comparison was made of samples of SHC from filter extracts, impactor plate extract, and aerosol generator waste, as well as whole-chamber atmosphere samples. No difference was observed in the chromatograms of four major samples. No peaks were observed from a chamber vapor atmosphere sample.

All male rats survived a 4-h inhalation exposure to 4.8 mg SHC/L; however, one of five female rats died within 48 h postexposure. During exposure the test animals demonstrated signs of eye and upper respiratory irritation. All surviving rats gained weight during the 14-day observation period. There was no statistical difference in body weight gain between the treatment groups and their corresponding control groups.

Gross observations at sacrifice failed to reveal any treatment-related lesions and lungs harvested for microscopic examination were unremarkable. Microscopic findings in the lungs of the rat that died included marked, diffuse congestion and edema.

### **DISCUSSION**

In the oral and dermal toxicity studies, no deaths or signs of toxicity were observed in any of the animals, and body weight gains during the subsequent 14-day observation period appeared to be unaffected by treatment. Inhalation of SHC at near-the-limit concentration produced irritation in all rats and death in one of 10 rats. Microscopic findings of congestion and edema in the lungs of the rat that died were typical of lesions related to lung and respiratory tract irritation. Microscopic examination of the lungs of the survivors failed to reveal marked exposure-related lesions. SHC exhibited a mild irritating effect to the conjunctival tissue of rabbit eyes. Rinsing the eyes after treatment was of questionable benefit. Remarkable irritating effects were not observed as a result of exposure to intact skin of rabbits nor did the repeat application elicit a sensitization reaction in guinea pigs.

Table 3.13-1 is a summary of the acute test results from exposure to SHC. Under the conditions of these tests, SHC did not demonstrate an acute toxicological hazard.

TABLE 3.13-1. SUMMARY OF ACUTE TEST RESULTS FOR SILAHYDROCARBON

Eye Irritation	Skin Irritation	Sensitization	Oral LD50 (g/kg)	Dermal LD50 (g/kg)	Inhalation LC <sub>50</sub> (mg/L)
Slight	Negative	Negative	> 5.0	> 2.0	> 4.8

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## SECTION 4

### PHARMACOKINETIC AND PHARMACODYNAMIC MODELING

#### 4.1 CLEARANCE OF ALANINE AMINOTRANSFERASE FROM BLOOD IN MICE AND RATS

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#### ABSTRACT

A physiologically based pharmacokinetic (PB-PK) model for chloroform-induced hepatic cytotoxicity and carcinogenicity presently is being developed. Hepatocellular necrosis and regeneration were incorporated into the model. Serum alanine aminotransferase (ALT) activity was chosen as an index of hepatocyte death. A description of blood ALT kinetics was included in the model. This study was conducted to determine the clearance rate of ALT from blood in mice and rats for validation of the PB-PK model. ALT activity was determined in liver and blood in control animals (mice: 84,650 and 93 U/L, respectively; rats: 75,434 and 64 U/L, respectively). Hepatic cytosol, containing high ALT activity, was injected intravenously into animals, and time-course curves of the disappearance of ALT from blood were fit with a monophasic exponential equation in rats and a biphasic exponential equation in mice. ALT clearance was twice as fast in mice as in rats. Half-life of injected ALT was 8 h in rats and 4 h in mice. The blood elimination rate constant of ALT in rats was 0.086 (1/h). In mice 84% of the injected ALT was cleared with a rate constant of 0.177 (1/h).

#### INTRODUCTION

Many chemicals identified by the Installation Restoration Program (IRP) as groundwater contaminants at Air Force installations are rodent carcinogens. Humans are exposed to these chemicals by ingestion of drinking water derived from contaminated groundwater. Chloroform is one such contaminant that is a known hepatocarcinogen. A PB-PK model based on Ramsey and Andersen (1984) and Andersen et al. (1987) is being developed for chloroform-induced hepatic cytotoxicity/carcinogenicity (Corley et al., In Press; Reitz et al., In Press). This model ultimately will be used for chloroform hepatocarcinogenic risk assessment in humans, and the basic model structure will be adapted for risk assessment of other IRP chemicals.

Hepatocyte death and regeneration have been incorporated into the PB-PK model. A noninvasive quantitative index of hepatic cytotoxicity, ALT, also was incorporated into the model. ALT, which is located in the cytoplasm of liver, heart, and skeletal muscle cells (Griffiths, 1979), leaks into the blood from damaged hepatocytes (Balazs et al., 1961, 1962; Schmidt, 1978; Tyson et al., 1983). Experimentally induced hepatic necrosis has been detected by increased serum ALT (Balazs et al., 1961, 1962), and a strong linear correlation between the percent of damaged hepatocytes and the

log of the peak serum ALT activity has been described (Hewitt et al., 1980). Therefore, elevated serum ALT activity may be used not only as an indicator of liver damage, but also as a measure of the extent of the necrotic lesion (Plaa and Hewitt, 1982). Thus, serum ALT was chosen as an index of the severity of liver necrosis for the PB-PK model. Blood ALT kinetics were described in the model. Little information was found in the literature concerning blood ALT clearance in rats or mice. The half-life of pig heart ALT was approximately 10 h when injected into mice (Massarrat, 1965). A biphasic equation for ALT elimination in dogs with half-times of 17 h (fast phase) and 60 h (slow phase) has been determined (Fleisher and Wakim, 1956, 1961, 1963). The half-life of ALT in human circulation is  $47 \pm 10$  h (Bar and Ohlendorf, 1970). The following study was performed to determine the clearance rate of cytosolic ALT from blood in rats and mice for validation of the PB-PK model.

## METHODS

### *Animals*

Male Osborne-Mendel rats, weighing approximately 250 g, were obtained from Camm Research Institute (Wayne, NJ). Male B6C3F<sub>1</sub> mice, weighing approximately 25 g, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were maintained under standard conditions of light (6:00 a.m. - 6:00 p.m.) and temperature (23 °C), and received water and food (Purina Lab Chow, Ralston Purina, St Louis, MO) ad libitum.

### *Cytosol Preparation*

Animals were sacrificed by cervical dislocation and the livers were removed quickly. Tissues were weighed and homogenized in three volumes of cold 0.9% NaCl Tris buffer solution (pH 7.4). After a series of centrifugations (Colby et al., 1980) culminating at 105,000 x g for 75 min in a Beckman L8-70 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA), cytosol was collected as the supernatant fraction.

### *Cytosol Injections*

Animals were anesthetized with 50% oxygen/50% carbon dioxide (Matheson Gas, Twinsburg, OH). Hepatic cytosol was injected into the femoral vein of the animals. Mouse liver cytosol was injected into mice in a volume of 100 µL at a concentration of 19,000 to 35,000 U/L. Five hundred microliters of rat liver cytosol (15,000 to 26,000 U/L) was injected into each rat. Blood was collected from the mice via the orbital sinus and from the rat via the lateral tail vein. Blood samples (80 µL in a heparinized microhematocrit tube) were collected at 5 min and 1, 6, 12, 24, 30, and 48 h postinjection from mice, and at 5 min and 2, 6, 8, 12, 24, 30, and 60 h postinjection from rats. Animals were anesthetized with 50% oxygen/50% carbon dioxide during blood collection. ALT activity in plasma

and hepatic cytosol was measured with a Kodak Ektachem 700XR analyzer (Eastman-Kodak, Rochester, NY) using the method of Bergmeyer and Horder (1980).

#### Statistics

Clearance rate constants were determined by nonlinear regression of the blood elimination data using RS/1 Release 3.0 software (BBN Software Products Corp., Cambridge, MA). Hepatic and serum ALT data were analyzed by Student's t-test;  $p < 0.05$  was considered significant.

#### RESULTS

Normal ALT activity in rats and mice is listed in Table 4.1-1. Blood ALT activity was similar in normal mice and rats, as was the case with hepatic ALT. In both species, ALT was approximately 1000 times higher in liver than in blood. Normal mouse plasma ALT data were highly variable despite the large sample size.

TABLE 4.1-1. ALT ACTIVITY (U/L) IN RATS AND MICE

Parameters	Plasma	Hepatic Cytosol
Rats		
Mean	64	75,434
SD	18	34,219
N	4	5
Mice		
Mean	93	84,650
SD	90	18,704
N	54	4

Clearance of ALT in mice (Figure 4.1-1) is described best with a biphasic equation (multiple  $R^2 = 0.99$ ) of the form  $A = a_1 e^{k_1 t} + a_2 e^{k_2 t}$ , in which A represents the plasma ALT activity at any point in time. The  $a_1$  equals the amount of ALT cleared with the rate constant  $k_1$  of the fast phase, and  $a_2$  is the amount of ALT cleared with the rate constant  $k_2$  of the slow phase. Half-times were calculated by dividing the rate constant by 0.693. The higher variability in serum ALT concentrations at the early time points seen in the mice may, in part, be due to the wider range in concentration of cytosolic ALT injected in the mice as compared to the rats. A biphasic equation for ALT elimination in the rat (Figure 4.1-2) was not necessary because the data could be described accurately with a monophasic equation (multiple  $R^2 = 0.999$ ) of the form  $A = a_1 e^{k_1 t}$ .

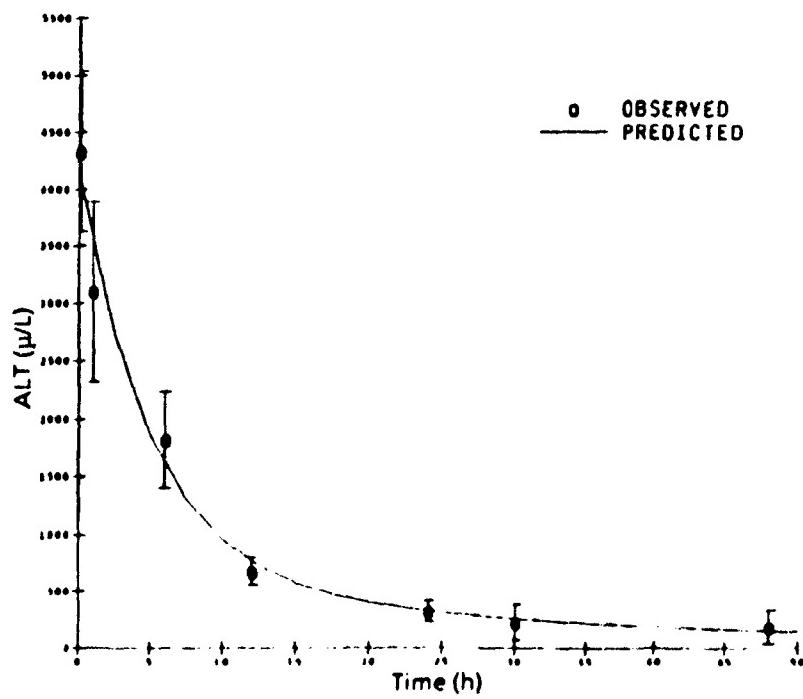


Figure 4.1-1. Clearance of ALT from Blood of Mice. Values are the mean  $\pm$  SD of four to eight animals

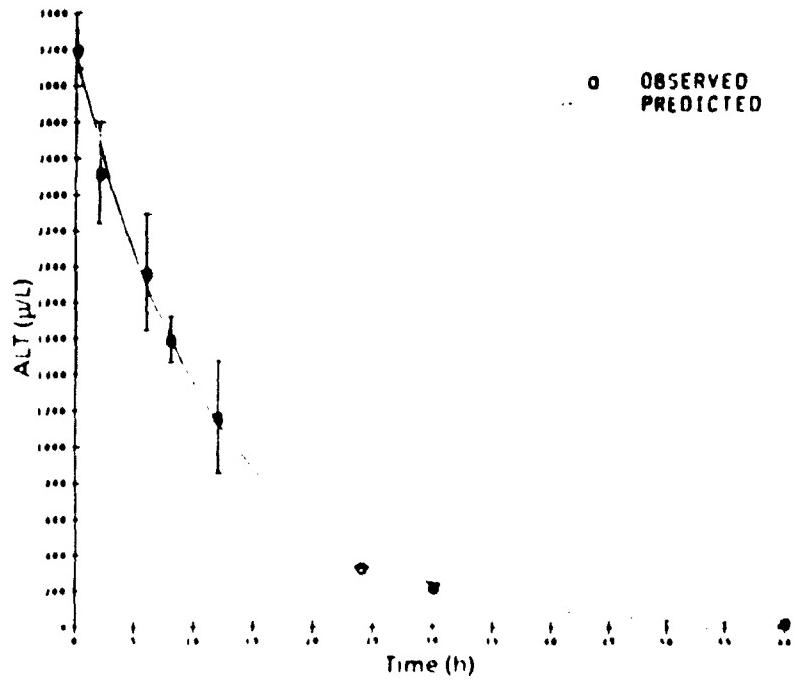


Figure 4.1-2. Clearance of ALT from Blood of Rats. Values are the mean  $\pm$  SD of four to eight animals

Table 4.1-2 lists the ALT half-life, clearance rate constants, and the percentage of injected ALT cleared per phase. The majority of injected ALT was eliminated twice as fast in mice as in rats. The elimination rate constants listed in Table 4.1-2 were determined by nonlinear regression of the clearance curves for ALT in mice (Figure 4.1-1) and rats (Figure 4.1-2).

TABLE 4.1-2. ALT HALF-LIFE AND CLEARANCE RATE CONSTANTS IN RATS AND MICE

Parameters	K (1/h)	HALF-LIFE (h)	% Cleared
Rats	0.086	8.06	100
Mice			
k	0.177	3.92	84
kk	0.028	24.75	16

#### DISCUSSION

The results presented herein describe the clearance of ALT from blood in mice as a two-compartment or biphasic process. In the data for rats, however, the blood elimination of ALT was accurately described as a monophasic process. Elimination of ALT was twice as fast in mice as in rats, with half-times of approximately 4 and 8 h, respectively. These data are in accord with previous data in the literature concerning ALT clearance from blood. Pig heart ALT was eliminated with a half-life of 10 h in mice (Massarrat, 1965). Fleisher and Wakim (1963) investigated ALT clearance in dogs after an iv injection of purified enzyme. A biphasic blood elimination curve was described with a rate constant of 0.012 (1/h) in the slow phase and 0.043 (1/h) in the fast phase. Corresponding half-times were calculated as 60.9 and 17.1 h, respectively. Sixty-three percent of the injected enzyme was cleared through the initial rapid phase, with the remainder being cleared in the slow phase.

In the present study, ALT clearance may have been altered by competition of other proteins present in hepatic cytosol for the clearance mechanism. However, Fleisher and Wakim (1956, 1961, 1963) observed similar rates of ALT clearance in dogs regardless of whether a crude or a purified preparation of ALT was injected. In addition, these investigators described a similar rate of ALT clearance in dogs that had been treated with carbon tetrachloride to induce liver damage and subsequent ALT release (Fleisher and Wakim, 1956). These data suggest that the deposition of other cytosolic liver proteins into the blood resulting from the toxic insult does not significantly alter the rate of ALT elimination.

The mechanism through which ALT is cleared from blood is unknown. Neither splenectomy nor blockage of the reticulo-endothelial system with India ink had an effect on ALT half-life in dogs

(Fleisher and Wakim, 1963). ALT was not excreted into the urine, suggesting that the kidney is not involved in removing ALT from the blood (Fleisher and Wakim, 1963).

ALT elevation has been shown to be a good indicator of liver damage by several investigators. Balazs et al. (1961) found a good correlation between the extent of liver necrosis and the elevation of serum ALT activity in rats treated with allyl alcohol. Hewitt et al. (1979 and 1980) found a very strong correlation between histologic changes in the liver and peak serum ALT activity in rats and in mice. In fact, Plaa and Hewitt (1982) suggested that not only can serum ALT be used to detect the presence of hepatic injury, but the severity of the lesion can be estimated by the degree of ALT elevation. Therefore, peak plasma ALT activity was chosen as an index of the extent of hepatocytic necrosis due to chloroform cytotoxicity for the PB-PK model. The ALT clearance rate constant values determined in this study will be used to validate the model description of blood ALT kinetics after chloroform insult.

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#### **4.2 CORRELATION BETWEEN CHLOROFORM-INDUCED HEPATIC NECROSIS AND PLASMA ALANINE AMINOTRANSFERASE ACTIVITY IN MICE**

**D.A. Staats, G.A. King, J.M. Gearhart, and R.B. Connolly**

##### **ABSTRACT**

The following investigation was performed to determine the relationship between plasma alanine aminotransferase (ALT) activity and hepatic necrosis due to chloroform (CHLF) cytotoxicity in mice. Mice were gavaged with 100, 200, or 500 mg/kg CHLF in corn oil. Blood ALT time-course data revealed that plasma ALT activity peaks 24 h postexposure. Mice were treated as described above and sacrificed at 24 or 48 h after exposure. Hepatic damage was assessed with quantitative light microscopy. Hepatocytes were graded as normal, degenerated, or necrotic. A linear correlation was found between the percentage of degenerated plus necrotic cells and plasma ALT ( $R^2 = 0.81$ ) at 24 h postexposure, and also between the percentage of necrotic cells 48 h after treatment and plasma ALT ( $R^2 = 0.88$ ) 24 h after treatment. The latter relationship was correlated more strongly. At 24 h most of the damaged cells were considered degenerated; whereas, at 48 h all damaged cells had pyknotic nuclei and were graded necrotic. The linear equations determined by regression of these two data sets were very similar. These results suggest that the damaged hepatocytes did not recover, but died slowly over a 48-h period. Plasma ALT activity 24 h after CHLF treatment appears to be a good quantitative index of the extent of hepatic necrosis due to CHLF cytotoxicity.

##### **INTRODUCTION**

The Installation Restoration Program (IRP) has identified numerous chemical contaminants in groundwater at Air Force installations. Many of these chemicals are rodent carcinogens. Humans are exposed to these chemicals by ingestion of drinking water derived from contaminated groundwater. CHLF, an IRP groundwater contaminant, is a rodent hepatocarcinogen. A physiologically based pharmacokinetic (PB-PK) model based on Ramsey and Andersen (1984) and Andersen et al. (1987) is being developed for CHLF-induced hepatic cytotoxicity/carcinogenicity (Corley et al., In Press; Reitz et al., In Press). Ultimately, this model will be used to estimate human health risks associated with CHLF exposure. In addition, the basic model will be adapted for risk assessment of other IRP chemicals.

A description of toxicant-induced hepatic cytotoxicity (cell damage and death) has been included in the PB-PK model. Numerous investigators have used elevation of serum ALT activity as an indicator of hepatic damage resulting from toxic insult (Schmidt, 1978). Balazs et al. (1962) measured allyl alcohol-induced hepatic necrosis using a planimetric method and found a strong linear correlation between the area of necrosis and the elevation in ALT activity. Tyson et al. (1983) used elevation of serum ALT activity to rank the relative hepatotoxicity of five haloalkanes in rats. Hewitt et al. (1979, 1980) used light microscopy to evaluate cellular damage resulting from CHLF

hepatotoxicity in rats and mice. In this study hepatocytes were graded as normal, degenerated, or necrotic. A strong linear correlation was determined between the elevation in serum ALT activity and the percentage of degenerated hepatocytes; however, no necrotic hepatocytes were observed in rats or mice treated with CHLF (0.5 mL/kg ip and 0.1 mL/kg po, respectively). The following study was performed to determine the relationship between CHLF-induced hepatocyte death and elevated plasma ALT activity. Information gleaned from this study will be used to validate the PB-PK model for CHLF cytotoxicity/carcinogenicity.

## METHODS

### *Animals*

Male B6C3F<sub>1</sub> mice weighing approximately 25 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were maintained under standard conditions of light (6 a.m. to 6 p.m.) and temperature (23 °C), and were given food (Purina Lab Chow, Ralson Purina Co., St. Louis, MO) and water ad libitum.

CHLF (Aldrich Chemical Co., Milwaukee, WI) was administered to mice by oral gavage at doses of 0, 100, 200, and 500 mg/kg in corn oil (final volume of 1 µL/g body weight). ALT time-course blood samples were obtained at 6, 12, 24, 30, and 48 h after CHLF treatment. In histopathological studies, blood samples were taken 24 and 48 h after CHLF exposure. All blood samples were taken from the orbital sinus with a heparinized microhematocrit tube (80 µL) under anesthesia with 50% oxygen/50% carbon dioxide. Animals were sacrificed by halothane overdose.

### *Histopathology*

Liver sections, taken at the bifurcation of the median lobe upon sacrifice at 24 or 48 h after CHLF exposure, were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Tissue sections were cut and stained with hematoxylin and eosin. Hepatocyte damage was quantified microscopically by scanning 25 randomly chosen fields at 400× magnification according to the method described by Chalkley (1943), Mitchell et al. (1973), and Hewitt et al. (1980). A micrometer was placed inside one eyepiece and 20 points of reference were chosen. In each of the 25 fields, the cell beneath each of the 20 points was graded as either normal, degenerated, or necrotic. Degenerated hepatocytes were characterized by increased cytoplasmic vacuolization and decreased basophilic staining. Necrotic hepatocytes were characterized by diminished basophilia, marked cytoplasmic vacuolization, and pyknotic nuclei. A total of 400 cells were counted and the three categories were expressed as a percentage of the total. One section was considered representative of the entire organ (Mitchell et al., 1973). Representative fields of liver sections were photomicrographed.

### Biochemistry

Livers were removed quickly from normal mice, weighed, and homogenized in three volumes of cold 0.9% NaCl Tris buffer solution (pH 7.4). Hepatic cytosol was collected as the supernatant fraction after the liver homogenate was subjected to a series of centrifugations (Colby et al., 1980) culminating at 105,000  $\times g$  for 75 min in a Beckman LB-70 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). ALT activity in plasma and in hepatic cytosol was determined with a Kodak Ektachem 700XR analyzer (Eastman Kodak, Rochester, NY) by the method of Bergmeyer and Horder (1980).

### RESULTS

Hepatic ALT activity was three orders of magnitude higher than plasma ALT activity in normal mice (Table 4.2-1). Plasma ALT data were highly variable despite the large sample size.

TABLE 4.2-1. ALT ACTIVITY (U/L) IN NORMAL MOUSE BLOOD AND LIVER

	Plasma	Hepatic Cytosol
Mean	93	84,650
SD	90	18,704
N	54	4

Plasma ALT activity was determined in mice up to 48 h after CHLF exposure (Figure 4.2-1). Peak plasma ALT activity occurred 24 h postexposure in each of the three dosage groups (100, 200, and 500 mg CHLF/kg).

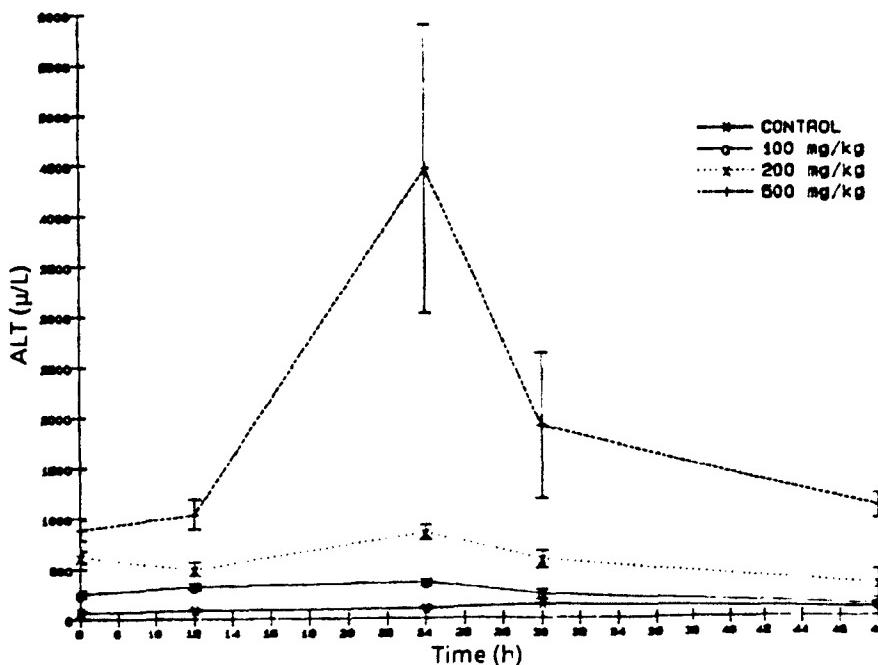


Figure 4.2-1. Time Course of Plasma ALT after Chloroform Exposure. Values are the means  $\pm$  SEM of eight mice.

Hepatocytes in liver sections obtained from mice that were treated with corn oil vehicle alone appeared normal at both 24 and 48 h (Figure 4.2-2A) after treatment. Hepatocytes in the centrilobular zone of mice sacrificed 24 h after treatment with 100, 200 (photomicrographs not shown), or 500 mg CHLF/kg (Figure 4.2-2B) exhibited cytoplasmic vacuolization, eosinophilic granularity, swelling, and diminished basophilia. The severity of these abnormal characteristics and the number of cells exhibiting these characteristics increased in a dose-dependent manner. The cytoplasm of damaged cells in liver sections from mice in the 500-mg CHLF/kg treatment group (Figure 4.2-2B) had a ground-glass appearance that was not evident in hepatocytes from animals in the two lower dose groups. Also, some hepatocytes (4%) in sections from mice in this dose group exhibited pyknotic nuclei and were considered necrotic. However, no necrotic cells were observed in sections from mice in the two lower dosage groups sacrificed 24 h after treatment (Table 4.2-2).

The objective of this study was to correlate CHLF-induced hepatocyte death with plasma ALT elevation. However, few necrotic cells were seen in mice sacrificed 24 h after CHLF treatment. Therefore, mice were treated as described above and sacrificed 48 h after treatment to allow time for the damaged cells to either (1) recover from injury or (2) exhibit cellular death characteristics (i.e., pyknotic nuclei).



Figure 4.2-2A. Liver from a Control Mouse 48 h after Treatment with Corn Oil Vehicle Only.  $\times 400$ .



**Figure 4.2-2B.** Liver from a Mouse 24 h after Treatment with 500 mg Chloroform/kg. Hypertrophy, vacuolization, and diminished basophilia of hepatocytes of the centrilobular zone are seen.  $\times 400$ .

**TABLE 4.2-2. HISTOLOGICAL EVALUATION<sup>a</sup> OF CHLOROFORM-INDUCED HEPATOTOXICITY**

Treatment	Normal Hepatocytes(%)	Degenerated Hepatocytes(%)	Necrotic Hepatocytes(%)
<b>24 h</b>			
Control	95.7 $\pm$ 1.5	4.3 $\pm$ 0.7	0
100 mg/kg	88.0 $\pm$ 2.1	11.8 $\pm$ 0.9	0
200 mg/kg	82.6 $\pm$ 1.5	17.4 $\pm$ 0.7	0
500 mg/kg	76.0 $\pm$ 4.6	20.0 $\pm$ 1.1	4.0 $\pm$ 1.1
<b>48 h</b>			
Control	98.1 $\pm$ 0.2	1.5 $\pm$ 0.1	0.4 $\pm$ 0.1
100 mg/kg	91.9 $\pm$ 0.3	0	8.1 $\pm$ 0.2
200 mg/kg	80.3 $\pm$ 1.1	0	19.7 $\pm$ 2.9
500 mg/kg	73.3 $\pm$ 7.4	0	26.7 $\pm$ 3.0

\* One liver section from each of six mice was evaluated.  
Values are means  $\pm$  SEM.

Hepatocytes in sections obtained from mice sacrificed 48 h after treatment with 100, 200 (photomicrographs not shown), or 500 mg CHLF/kg (Figure 4.2-2C) exhibited abnormal characteristics similar to those seen in the 24-h sacrifice groups. However, these cells also had pyknotic nuclei and were graded necrotic. No degenerated cells were observed in these sections (Table 4.2-2).



**Figure 4.2-2C.** Liver from a Mouse 48 h after Treatment with 500 mg Chloroform/kg. Hypertrophy, marked vacuolization, diminished basophilia, and pyknotic nuclei of hepatocytes of the centrilobular zone are seen.  $\times 400$ .

Average percentages of normal, degenerated, and necrotic hepatocytes observed in liver sections from mice treated with different doses of CHLF and sacrificed 24 or 48 h after treatment were summarized (Table 4.2-2). In the 24-h sacrifice group, most damaged hepatocytes were degenerated not necrotic. Necrotic hepatocytes were observed only in the sections from mice in the highest dose group (500 mg/kg). However, in sections from mice sacrificed 48 h after treatment, most damaged hepatocytes were graded as necrotic not degenerated. Degenerated cells were observed only in sections from control mice. No statistically significant difference in the percentage of degenerated plus necrotic cells was seen between corresponding dose groups in the two sacrifice groups (24 and 48 h after treatment).

Average plasma ALT activity was determined 24 h after CHLF treatment in both sacrifice groups (24 h and 48 h posttreatment) (Table 4.2-3). ALT activity increased in a dose-dependent manner in

both sacrifice groups. No statistically significant difference in plasma ALT 24 h after CHLF treatment was observed between corresponding dose groups in the two sacrifice groups.

TABLE 4.2-3. PLASMA ALT ACTIVITIES<sup>a</sup> (U/L) 24 H AFTER CHLOROFORM TREATMENT

Treatment	24-h Sacrifice	48-h Sacrifice
Control	68.8 ± 11.1	76.5 ± 4.1
100 mg/kg	129.3 ± 18.2	141.8 ± 21.1
200 mg/kg	251.0 ± 31.2	351.3 ± 9.6
500 mg/kg	2372.2 ± 1034.0	1407.8 ± 363.8

\* Values are means ± SEM.

Figure 4.2-3 is a graphic representation of the relation of the log of plasma ALT activity 24 h post-CHLF treatment (0, 100, 200, or 500 mg/kg) with respect to the percentage of damaged hepatocytes at 24 h posttreatment in individual mice. The percentage of damaged cells equals the percentage of degenerated cells plus the percentage of necrotic cells. A good correlation in these data was determined by linear regression ( $R^2 = 0.81$ ). The equation for the line of regression was  $Y = 0.04(X) + (1.56)$ .

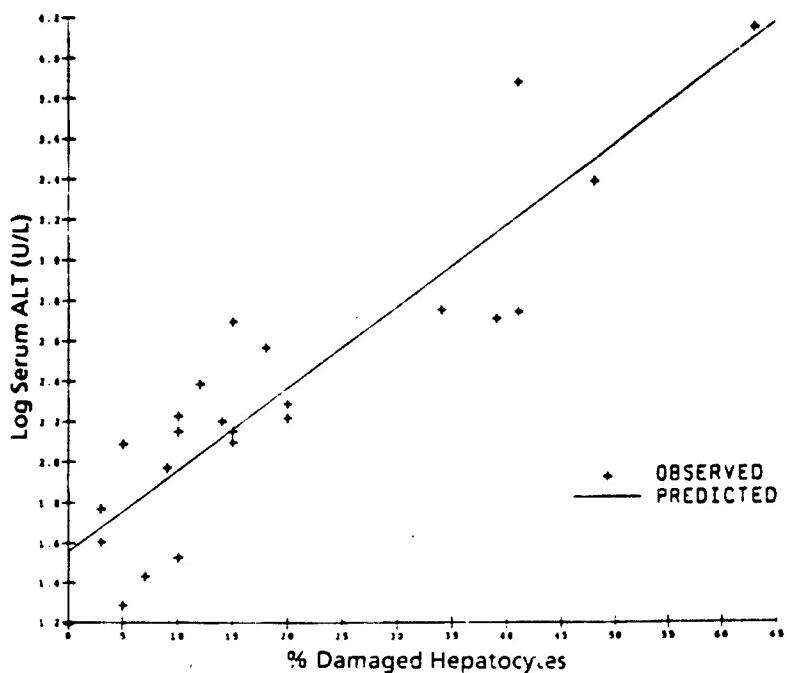


Figure 4.2-3. Log Plasma ALT Activity at 24 h Post-Chloroform Exposure (0, 100, 200, or 500 mg/kg) Related to Percentage of Damaged Hepatocytes at 24 h Post-Chloroform Exposure. The percentage of damaged cells equals percentage of degenerated plus necrotic cells. ( $r^2 = 0.81$ ).

Figure 4.2-4 graphically illustrates the relationship between the log of plasma ALT activity 24 h post-CHLF treatment (0, 100, 200, or 500 mg/kg) and the percentage of necrotic hepatocytes 48 h posttreatment in individual mice. A stronger correlation was determined in these data than in the data presented in Figure 4.2-3 ( $r^2 = 0.88$  and  $r^2 = 0.81$ , respectively). The equation of the line of correlation for these data was  $Y = 0.04(X) + (1.78)$ . The linear equations of the correlation for the data in Figures 4.2-3 and 4.2-4 are very similar. This suggests that the degenerated cells observed 24 h post CHLF treatment could not recover from the toxic damage and were dead 48 h after treatment. These data indicate that peak plasma ALT activity after CHLF insult is a good quantitative index of hepatocellular damage and death.

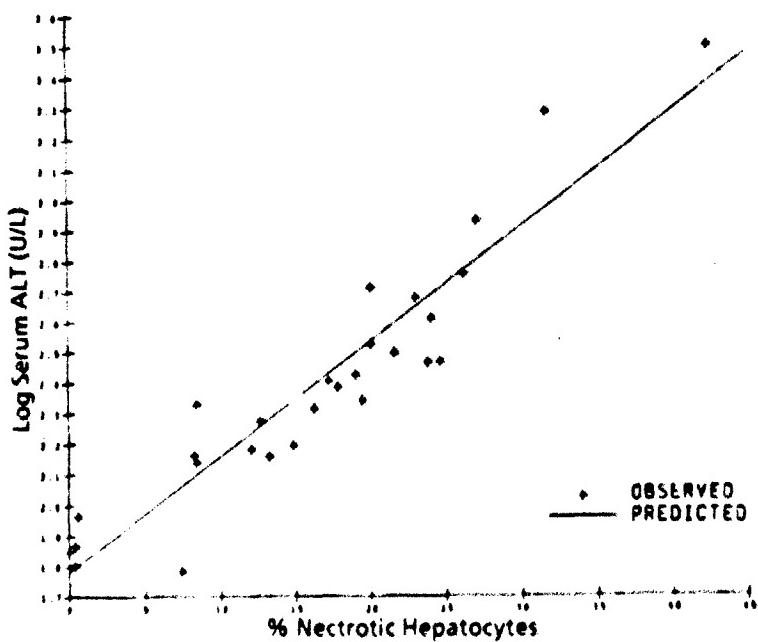


Figure 4.2-4. Log Plasma ALT Activity at 24 h Post-Chloroform Exposure (0, 100, 200, or 500 mg/kg) Related to Percentage of Necrotic Hepatocytes at 48 h Post-Chloroform Exposure. ( $r^2 = 0.88$ ).

#### DISCUSSION

The results contained in this report suggest that elevation of plasma ALT activity is a good index of CHLF-induced hepatotoxicity for the PB-PK chloroform cytotoxicity/carcinogenicity model. These findings are in accord with those determined by previous investigators. In 1962 Balazs et al planimetrically measured the surface area of necrotic lesions in hepatic lobes from mice treated with 2% allyl alcohol. A good correlation ( $r^2 = 0.874$ ) was found between the area of the necrotic lesion and the serum ALT activity 24 h after treatment. Tyson et al (1983) ranked the relative hepatotoxicity of five haloalkanes, including CHLF, by the dose that produced an above normal serum ALT level in

50% of the rats tested. The resulting relative potency ranking of the chemicals was identical to the ranking determined by these investigators in an *in vitro* hepatocyte system (Tyson et al., 1983). In both the present study and the Tyson et al. (1983) study, the peak blood ALT activity induced by a single oral dose of CHLF occurred 24 h after treatment.

Hewitt et al. (1979) evaluated CHLF-induced hepatocellular damage in mice with light microscopy. Hepatocytes were graded as normal, degenerated, or necrotic (as in the present study). Liver sections from mice treated with 148 mg CHLF/kg exhibited 8.8% ( $\pm$  2.6 SE) degenerated cells 24 h after treatment. However, no necrotic cells were observed in these sections. Necrotic cells (28%  $\pm$  11.0 SE) were observed in liver sections taken from mice 24 h after treatment with a very large dose of CHLF (1483 mg/kg). Degenerated cells (58.6%  $\pm$  5.1 SE) also were observed in these sections. In 1982 Plaa and Hewitt used the data from this study to demonstrate a strong correlation ( $r = 0.96$ ) between the log of serum ALT and the percentage of damaged (degenerated plus necrotic) hepatocytes 24 h after CHLF treatment. However, some of these data were collected from animals that were treated with kepone, a potentiating agent, in addition to being treated with CHLF. The objective of the study presented herein was to determine the above correlation in mice treated with CHLF only so that the possible influence of other factors, such as a potentiating agent, would be negated.

The results of the Hewitt et al. (1979) study are very similar to the results of the present study. In both studies, no necrotic cells were observed in hepatic sections from mice sacrificed 24 h after treatment with a single low dose of CHLF. Also, the average percentage of degenerated cells was similar in sections from mice treated with similar doses of CHLF in the two studies even though different strains of mice were used (see Table 4-2-2). Swiss-Webster mice were used in the Hewitt study. Because B6C3F<sub>1</sub> mice were used in the National Cancer Institute CHLF bioassay (1976), this strain was chosen for use in the present study. In addition, the linear equations of the correlations between ALT and damaged hepatocytes 24 h after CHLF treatment in the Hewitt study and in the present study are remarkably similar ( $Y = 0.04[X] + [1.55]$  and  $Y = 0.04[X] + [1.56]$ , respectively).

The goal of this study was to correlate CHLF-induced plasma ALT elevation with hepatocyte death. Because no dead hepatocytes were observed in sections from mice sacrificed 24 h after low-dose CHLF treatment in either the Hewitt et al. (1979) study or the present study, the duration of exposure before sacrifice was increased. This increase allowed time for the damaged cells to either (1) recover from the toxic insult or (2) exhibit characteristics indicative of cellular necrosis (i.e., pyknotic nuclei). Sections obtained from mice sacrificed 48 h after CHLF treatment exhibited no degenerated hepatocytes. However, necrotic cells were observed in these sections. In fact, the average percentage of necrotic cells found in these sections were very similar to the average percentage of degenerated cells found in sections from mice sacrificed 24 h after treatment with

CHLF at equal doses. Also, the linear equation for the correlation of ALT with cellular damage 24 h after CHLF treatment was very similar to the linear equation determined in data from mice sacrificed 48 h after CHLF treatment ( $Y = 0.04[X] + [1.56]$  and  $Y = 0.04[X] + [1.78]$ , respectively). These data suggest that hepatocytes that can be histopathologically identified as damaged 24 h after CHLF treatment cannot recover from toxic injury and are destined to die. In conclusion, plasma ALT elevation 24 h after CHLF treatment would be a good quantitative index for the PB-PK model of CHLF-induced hepatocyte death.

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#### **4.3 EFFECT OF AGING ON CHLOROFORM METABOLISM AND TISSUE SOLUBILITY**

**D.A. Staats, G.A. King, and R.B. Conolly**

##### **ABSTRACT**

Tissue partition coefficients and metabolic constants for a given chemical are integral components of physiologically based pharmacokinetic (PB-PK) models. Recently, PB-PK models have been utilized in the risk assessment process. Frequently, the data base for risk estimations is a bioassay study that extends over the rodent's lifetime. Commonly, the effect of aging on the physiology of the animals has not been considered in the risk assessment. This study was conducted to determine the effect of aging on tissue partition coefficients and the rate of *in vivo* metabolism of chloroform (CHLF) in rats and mice. Little difference in CHLF coefficients was observed between young and old mice or rats. Young and old rats metabolized CHLF at a similar rate. However, the metabolic rate constant ( $V_{max}$ ) was threefold lower in elderly mice than in young mice. These results suggest that aging had little discernible impact on the solubility of CHLF in the body tissues of mice or rats. Also, the effect of aging on CHLF metabolism is species specific - CHLF metabolism is reduced in aged mice but not in aged rats.

##### **INTRODUCTION**

A partition coefficient for a given chemical is the ratio of the chemical concentration at equilibrium in two different media. The partition coefficient is an index of the relative solubility of the chemical in the two media. Blood:air and tissue:blood partition coefficients are required in PB-PK models to describe the uptake and distribution of the chemical in the body. PB-PK models also require kinetic constants to describe the metabolism of the chemical by the body. Metabolism may involve a Michaelis-Menten (saturable)-type kinetic process and/or a first-order process. Recently, PB-PK models have been utilized in the determination of carcinogenic risk to estimate the target tissue dose (Andersen et al., 1987; Reitz et al., In Press). Many risk estimations are based on tumor data from National Cancer Institute (NCI) bioassay studies. These studies are conducted over the entire life span of the rodent. At present, the effect of aging on the solubility of tissue:blood partition coefficients has not been described. Also, no general statements can be made concerning the effect of aging on metabolism (Birnbaum, 1987). Thus far, age-related metabolic changes that have been described are compound, species, strain, and sex specific (Birnbaum, 1987).

The following study was conducted to determine the effect of aging on tissue solubility and *in vivo* metabolism of CHLF. Information from this study ultimately will contribute to the development of a PB-PK model for CHLF cytotoxicity/carcinogenicity.

## METHODS

### Animals

Male Osborne-Mendel rats were obtained from Camm Research Institute (Wayne, NJ). Two age groups of rats were used in this study: (1) young adult (7-to-10-week-old, 250 g), and (2) aged adult (18-to-20-month-old, 600 g). Male B6C3F<sub>1</sub> mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Two age groups of mice were used: (1) young adult (7-to-10-week-old, 25 g), and (2) aged adult (18-to-20-month-old, 50 g). Aged rats were purchased from the supplier at one year of age and housed to the desired age. Aged mice were purchased from the supplier at five weeks of age and housed to the desired age for experimentation. All animals were maintained under standardized conditions of light (6 a.m. to 6 p.m.) and temperature (20 to 23 °C) and received food (Purina) and water ad libitum.

### Determination of Partition Coefficients

Animals were anesthetized with sodium pentobarbital (0.1 mL/100 g body weight) and blood was collected from the abdominal aorta with a heparinized syringe. Animals then were sacrificed by cervical dislocation and liver, kidney, thigh muscle, and fat (epididymal and perirenal) tissues were removed.

Tissues were homogenized in 0.9% NaCl (saline) solution in a 1:3 tissue weight:saline volume ratio. CHLF (Aldrich Chemical Co., Milwaukee, WI; 99.9% pure) partition coefficients were determined using the method of Gargas et al. (1988b, 1989), which is based on the vial-equilibration techniques of Sato and Nakajima (1979) and Fiserova-Bergerova et al. (1984). Young and old animals were compared in each species and in corresponding age groups between species using Student's t-test;  $p < 0.05$  was considered significant.

### Gas Uptake Studies

A closed, recirculated system described previously by Gargas et al. (1986a) was used to expose animals to various initial concentrations of CHLF. Individual rats were exposed to starting atmospheric concentrations of 350, 500, 1000, and 2000 ppm CHLF in a 2.7-L chamber. Individual mice were exposed to initial concentrations of 1000, 2000, and 3000 ppm CHLF in a 0.84-L chamber. Because an individual animal/small chamber was used as opposed to a multiple animal/large chamber (Gargas et al., 1986a, 1986b, 1988a), an ice bath around the outside of the chamber for condensation of exhaled water vapor was not necessary. Also, the possibility of reduced animal body temperature due to the ice was eliminated. The chamber system was dried with circulated room air after each exposure. The atmospheric CHLF concentration was analyzed 5 min after injection of the chemical and every 15 min thereafter during exposure by automatic injection into a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph (with a 3392A integrator). The chromatograph was equipped with a hydrogen flame ionization detector (250 °C), a 6-ft long, 1/8 in. o.d. stainless steel column packed

with 80/100 mesh Carbopack C, 0.1% SP 1000 (Supelco, Bellfonte, PA). Oven temperature was held constant at 120 °C.

#### **PB-PK Model and Data Analysis**

The metabolic constants were derived by gas-uptake data analysis using the computer program described previously by Gargas et al. (1986a), which is based on the model developed by Ramsey and Andersen (1984). The PB-PK computer model described the composition of the body as four tissue groups: liver, visera, muscle/skin, and fat. The change in CHLF concentrations in the four tissue compartments and in the exposure chamber compartment is described by mass balance differential equations that incorporated compartment volumes, blood flow to tissues, and tissue partition coefficients in the model. These equations are presented in detail by Gargas et al. (1986a) and Ramsey and Andersen (1984). The kinetic constants for metabolism ( $V_{max}$  and  $K_m$ ) were estimated using the PB-PK model to analyze the set of gas-uptake curves obtained for each animal group. The model was exercised using various values for the kinetic constants until a reasonable visual fit was achieved that described the data with a single set of constant values. Then SimuSolv, a FORTRAN-based computer software package (Dow Chemical Co., Midland, MI), was used to further refine the visually chosen  $V_{max}$  (mg/h/kg) and  $K_m$  (mg/L) using relative least squares weighting of the data. Student's t-test ( $p < 0.05$ ) was used to analyze the mean  $V_{max}$  for individually optimized uptake curves of each animal in young versus old groups.

#### **RESULTS**

Values used for ventilation, organ volumes, and blood flows (Table 4.3-1) were similar to those used previously in PB-PK models (Gargas et al., 1986a; Andersen et al., 1987; Corley et al., In Press; Reitz et al., In Press; Dawson, 1970). Liver weight was measured postexposure in CHLF gas-uptake experiments. No significant difference in liver weight (expressed as a percentage of body weight) was observed between young and old animals. Based on literature data and modeling observations (Novak, 1972; Lesser et al., 1973; Birnbaum, 1983; Gargas et al., 1986a), the percentage of body fat in young animals was doubled in senescent animals. An adequate fit of the gas-uptake data in senescent animals (Figures 4.3-1b and 4.3-2b) was obtained using these values for fat content (Table 4.3-1).

No statistically significant difference was detected in the respective tissue partition coefficients between young and old rats (Table 4.3-2). These values are very similar to those determined previously by Gargas et al. (1989) in the Fischer 344 (F-344) rat. The partition coefficient of CHLF in muscle tissue was significantly greater in old mice than in young mice (Table 4.3-2). Also, the muscle coefficient was significantly greater in old mice than in old rats. However, the fat partition coefficient was significantly greater in the young rat than in the young mouse. In accord with the Gargas et al. (1989) study, CHLF was far more soluble in fat tissue than in other tissues. The corresponding partition coefficients listed in Table 4.3-2 were incorporated into the PB-PK model during the analysis of the following gas-uptake studies.

TABLE 4.3-1. PARAMETERS USED IN THE PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR CHLOROFORM

Parameters	Young Rats	Old Rats	Young Mice	Old Mice
<b>Tissue weights<sup>a</sup></b>				
Liver <sup>b</sup>	4	4	6	6
Rapidly perfused	5	5	3	3
Slowly perfused	75	68	70	58
Fat	7	14	12	24
<b>Flows (L/h)<sup>c</sup></b>				
Alveolar ventilation	14	14	30	30
Cardiac output	14	14	30	30
<b>Blood flow<sup>d</sup></b>				
Liver	25	25	25	25
Rapidly perfused	51	51	51	51
Slowly perfused	15	15	15	15
Fat	9	9	2	2

<sup>a</sup> Expressed as a percentage of body weight.

<sup>b</sup> Parameters correspond to the average body weight in gas-uptake studies.

<sup>c</sup> Values are scaled to a 1.0-kg animal.

<sup>d</sup> Expressed as a percentage of cardiac output.

TABLE 4.3-2. CHLOROFORM PARTITION COEFFICIENTS<sup>a</sup>

Tissue	Young Rat	Old Rat	Young Mouse	Old Mouse
Blood	15.3 ± 0.7	22.1 ± 2.1	24.5 ± 2.6	26.7 ± 1.7
Liver	18.0 ± 1.3	14.5 ± 4.2	18.0 ± 1.2	29.3 ± 3.4
Kidney	12.5 ± 0.7	10.2 ± 1.3	17.2 ± 1.6	17.5 ± 2.3
Fat	340.9 ± 6.7	284.8 ± 14.9	173.3 ± 17.2 <sup>b</sup>	283.2 ± 17.1
Muscle	13.9 ± 1.6	11.9 ± 2.5	17.2 ± 2.6	101.7 ± 4.7 <sup>c,d</sup>

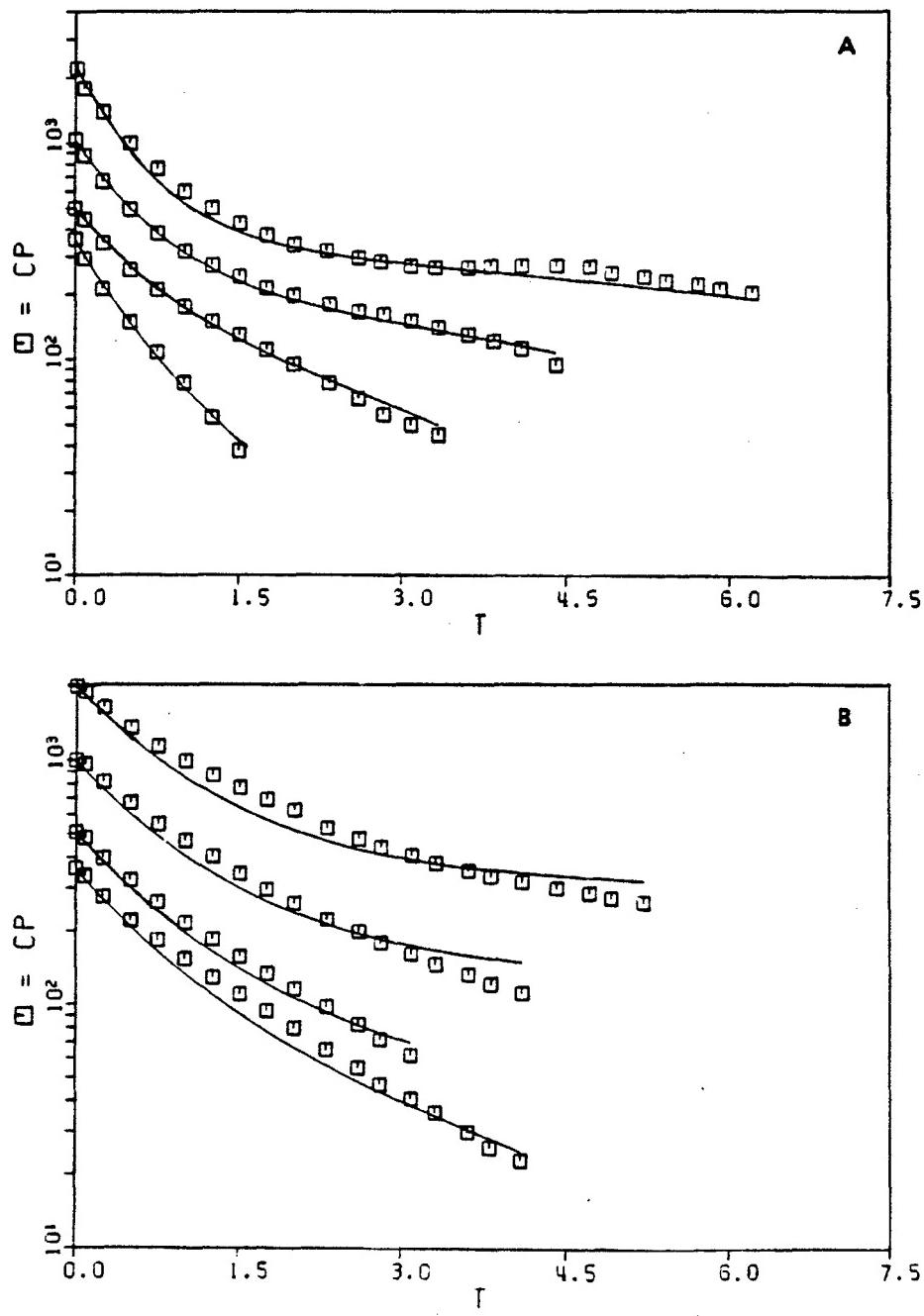
<sup>a</sup> Values expressed as means ± SEM of four to nine animals.

<sup>b</sup> p<0.05 vs. young rat value.

<sup>c</sup> p<0.05 vs. old rat value.

<sup>d</sup> p<0.05 vs. young mouse value.

Closed-chamber exposures of naive young (Figure 4.3-1a) and old rats (Figure 4.3-1b) to CHLF were conducted at initial concentrations of 350, 500, 1000, and 2000 ppm. Gas-uptake data for three individual rats per initial concentration were averaged (Figures 4.3-1a and 4.3-1b). The behavior of the uptake data was described adequately by a PB-PK model with a single, saturable metabolic pathway. Values for kinetic constants were determined by visual and SimuSolv optimization to best fit the data. Optimal values for V<sub>max</sub> and Km were 3.2 and 0.25 in young rats and 5.3 and 0.25 in old rats, respectively (Table 4.3-3). These values are similar to those published by Gargas et al. (1988b) for CHLF metabolism in young F-344 rats (V<sub>max</sub> = 7.0 and Km = 0.25).



**Figure 4.3-1.** Uptake of CHLF by (a) Young and (b) Old Rats Exposed at Initial Chamber Concentrations of 350, 500, 1000, and 2000 ppm. Solid lines are the curves generated by the computer model after visual and SimuSolv optimization of the kinetic constants, which gave the best representation of the data. Exposures were conducted with individual rats in a 2.7-L chamber. Points are the means for data of three rats exposed at each initial concentration. (Constants are listed in Table 4.3-3.)

TABLE 4.3-3. METABOLIC CONSTANTS IN YOUNG AND OLD RATS AND MICE<sup>a</sup>

Animal	V <sub>max</sub> <sup>b</sup>	V <sub>max</sub> <sup>c</sup>
<b>Rats</b>		
Young	3.2	5.3 ± 1.2
Old	5.3	5.4 ± 1.2
<b>Mice</b>		
Young	21.6	22.7 ± 1.8
Old	7.1	7.6 ± 0.7d

<sup>a</sup> Values expressed in milligrams per hour and scaled to a 10-kg animal.

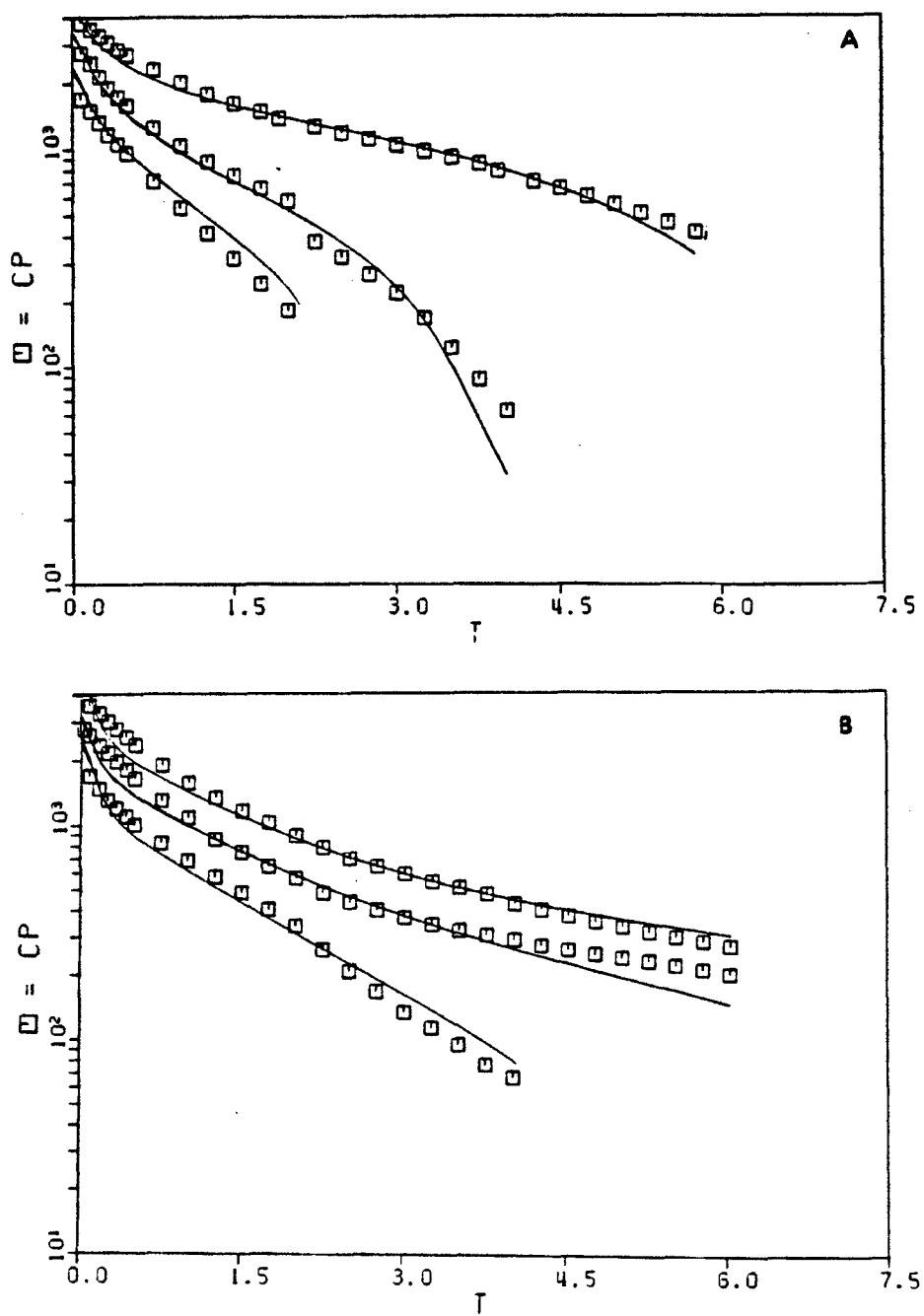
<sup>b</sup> Values are optimized for mean uptake data of three individually exposed animals per initial concentration (Figures 4.3-1 and 4.3-2).

<sup>c</sup> Values expressed as means ± SEM of individually optimized uptake data from nine animals.

<sup>d</sup> p<0.05 vs. corresponding young mice value.

Young (Figure 4.3-2a) and old (Figure 4.3-2b) mice were exposed to CHLF in a closed-chamber system at initial concentrations of 2000, 3000, and 4000 ppm. Data from three individual mice per initial concentration were averaged (Figures 4.3-2a and 4.3-2b). Kinetic constant values were optimized to best fit the family of uptake curves. The data were described adequately with a single, saturable metabolic pathway. Optimal values for V<sub>max</sub> and Km were 21.6 and 0.25 for young mice and 7.1 and 0.25 for old mice, respectively (Table 4.3-3). The uptake curves for old mice were simulated with the muscle partition coefficient value of the young mice. An adequate fit of the data could not be obtained with the muscle coefficient value determined in old mice (simulations not shown) (Table 4.3-2).

Gas-uptake data generated during each exposure of individual animals were simulated using the model. Metabolic parameters were optimized to best fit the individual curves. The V<sub>max</sub> values determined for each animal were averaged for each exposure group (young rats, old rats, young mice, and old mice) (Table 4.3-3). No significant difference in metabolism was observed between young and old rats. However, the mean V<sub>max</sub> in young mice was approximately threefold greater than in old mice. Also, these metabolic constant values were very similar to those determined by optimization of the average data for three individual animals exposed per initial concentration (Figures 4.3-1a, 4.3-1b, 4.3-2a, and 4.3-2b) (Table 4.3-3).



**Figure 4.3-2.** Uptake of CHLF by (a) Young and (b) Old Mice Exposed at Initial Chamber Concentrations of 1000, 2000, and 3000 ppm. Solid lines are computer-model-generated curves after optimization of kinetic constants. Exposures were conducted with individual mice in a 0.84-L chamber. Values are means of uptake data of three mice exposed at each initial concentration. (Constants are listed in Table 4.3-3.)

## **DISCUSSION**

The work presented herein suggests that tissue partition coefficients for CHLF do not change appreciably with age in mice or rats. Only the muscle coefficient was significantly different in senescent mice compared to young mice. However, the experimental CHLF gas-uptake data in senescent mice could not be simulated adequately using the muscle coefficient determined in mice of the same age. Use of the muscle coefficient value for young mice allowed an adequate fit of the uptake data in old mice. The increase in CHLF solubility in the muscle of senescent mice may be due to increased fat content in the muscle tissue with age. It is well known that body fat increases with aging (Novak, 1972; Lesser et al., 1973; Birnbaum, 1983, 1989). A possible reason for the modeling discrepancy is that the increase in the fat compartment already had been taken into account in the model. Fat content in aged animals in the model was twice that of young animals. The increase in muscle partition coefficient was not necessary in the model because of the increased fat compartment. Therefore, the increased muscle coefficient could be an artifact of increased fat in the muscle.

Generally, corresponding tissue partition coefficients for CHLF were similar in mice and rats. However, the fat coefficient in young rats was significantly higher than in young mice. Also, no strain difference in CHLF partition coefficients was observed between Osborne-Mendel rats (used in this study and in the NCI CHLF bioassay, 1976) and F-344 rats (Gargas et al., 1989).

In both mice and rats the metabolism of CHLF was described adequately using a PB-PK model with a single, saturable metabolic pathway (Michaelis-Menten kinetics). No indication of a first-order metabolic process was observed (Gargas and Andersen, 1988a). In the present study, *in vivo* CHLF metabolism in Osborne-Mendel rats was not discernibly affected by aging. Also, the rate of CHLF metabolism in these rats was similar to that determined previously in F-344 rats (Gargas et al., 1988b). In contrast, DiRenzo et al. (1982) found a 50% decrease in CHLF *in vitro* metabolism in livers of senescent F-344 rats. However, metabolism was not measured directly in this study. Instead, covalent binding to microsomal protein was used as an index of metabolism. Therefore, the decrease in macromolecular binding in senescent rats could have been due to a decrease in the target macromolecule with aging.

In summary, CHLF tissue solubility does not change appreciably with age in mice or rats. Also, the effect of aging on CHLF metabolism appears to be species specific.

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#### **4.4 DEVELOPMENT OF AN INITIATION/PROMOTION ASSAY TO DETECT FOCI OF ENZYME-ALTERED HEPATOCYTES**

**C.S. Godin and H.G. Wall**

##### **ABSTRACT**

Initiation with the potent carcinogen diethylnitrosamine (DEN) followed by promotion with phenobarbital (PB) resulted in detectable foci at the end of six weeks. Quantitation of these foci by image analysis stereological techniques indicated that staining for gamma-glutamyltranspeptidase (GGT)-positive foci was the best marker for the presence of enzyme-altered foci produced in response to a genotoxic carcinogen.

##### **INTRODUCTION**

The progression of biochemical changes that occurs following the transformation of a normal cell has been poorly understood. There have been nine well-documented experimental protocols that have been developed as a screen for hepatocarcinogens (Farber and Sarma, 1987). One of these models has described a two-stage development of hepatocarcinogenesis in which a limited exposure to a potential carcinogen (initiation) was followed by a prolonged exposure to a second agent (promotion)(Peraino et al., 1973). Evidence has indicated that stimulation of cellular proliferation is required before the initiated cell can be detected (Cayama et al., 1978; Columbano, 1981), resulting in the occurrence of enzyme-altered foci (EAF).

Several protocols describing chemically induced hepatocarcinogenesis have been shown to produce foci of initiated/promoted cells. One such method described by Pitot et al. (1978) and Sirica et al. (1978) has shown that, following partial hepatectomy to induce cell proliferation, initiation with a single dose of DEN followed by several weeks of promotion with PB resulted in an increased prevalence of altered foci, usually measured as GGT positive foci. Only those animals receiving both DEN and PB developed hepatocellular carcinomas, thereby clearly distinguishing between the initiation and promotion stages in hepatic carcinogenesis.

GGT-positive foci have been used widely as a marker for the detection of enzyme-altered foci and appear to be a dependable marker for enzyme-altered foci in the rat liver (Pugh and Goldfarb, 1978). Although GGT-positive foci have been used widely, other enzyme markers have been used such as adenosine triphosphatase (ATPase)-deficient foci, glucose 6-phosphatase (G6Pase)-deficient foci, and foci with decreased ability to accumulate iron. Recently, the placental form of rat glutathione S-transferase (GST-P) was shown to be elevated markedly in liver following the administration of DEN (Sato et al., 1984), and more recently single cells containing elevated GST-P levels were detected within 48 h of the administration of DEN (Moore et al., 1987).

In addition to the histochemical markers discussed, changes in certain enzyme activities have been shown following promotion. The induction of ornithine decarboxylase (ODC) has been associated with tumor promotion in the liver (Olson and Russell, 1980). Induction of ODC activity has been demonstrated to be a useful enzymatic marker for tumor promotion. Changes in peroxisomal enzymes involved in lipid  $\beta$ -oxidation have been shown to occur following exposure to such compounds as hypolipidemic agents and industrial plasticizers. Some of these compounds have been reported to produce liver tumors in rats (Lalwani et al., 1981; Reddy et al., 1980).

The following study was designed to establish and verify an initiation/promotion model based on the techniques described by Parnell (1986).

#### MATERIALS AND METHODS

##### *Animals*

Male Sprague-Dawley rats (three weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). They were quality control tested and found to be in acceptable health. The animals were group housed (four/cage) for one week in plastic cages containing hardwood-chip bedding and given a commercial diet (Purina Formula 5008) and water ad libitum.

##### *Experimental Regimen*

Animals were subjected to two-thirds partial hepatectomy using the procedure of Higgins and Anderson (1931) or were sham-hepatectomized. Twenty-four hours later, animals in the hepatectomized group received intraperitoneal injections of DEN (10 mg/kg body weight, Sigma Chemical Co., St. Louis, MO) in saline, while the sham group received injections of saline (1 mL/kg). Two weeks following this injection, hepatectomized animals were given PB (0.05% in the drinking water, Sigma Chemical Co., St. Louis, MO) for the next four consecutive weeks. At the conclusion of the study all animals were euthanized by CO<sub>2</sub> asphyxiation. The terminal animal and liver weights were obtained from each animal.

##### *Histological and Histochemical Studies*

Immediately after the animal's death, the liver was excised and weighed and the lobes were bisected. Sections of the right anterior and posterior lobes were taken for fixation in the following fixatives: buffered neutral formalin for routine hematoxylin and eosin (H&E) staining, alcoholic buffered formalin for glycogen staining, and acetic acid/formalin containing sodium sulfate for iron staining. Following fixation, each piece of tissue was embedded in paraffin and a 5- $\mu$ m section was prepared from three separate areas within each block of tissue and stained as follows. Routine H&E staining was performed on tissue submitted for fixation in buffered neutral formalin. Pieces of tissue fixed in alcoholic formalin were stained for the presence of glycogen using the periodic acid/Schiff

(PAS) reaction described by Bedi and Horobin (1976). Iron staining was accomplished using the Prussian blue procedure described by Hirota and Williams (1979).

A separate piece of liver from each of the lobes described above was frozen and six serial frozen sections (10  $\mu$ m thick) were prepared from three separate areas within each piece of liver. Adjacent sections from three different areas within each of the blocks of tissue were stained according to the procedures listed below. One section was stained for the presence of GGT activity using the method described by Rutenburg et al. (1969). An adjacent section was stained for the presence of ATPase activity according to the method described by Wachstein and Meisel (1957). Another adjacent section also was stained for the presence of G6Pase activity by the method described by Wachstein and Meisel (1958).

#### *Image Analysis*

Following staining and coverslipping, all slides were examined for the presence of foci. All foci were counted directly, using a HIPAD digitizing tablet (Houston Instruments, Austin, TX) coupled optically to the microscope, and were identified as those areas containing nine or more nuclei. The tissue area, number of foci, and foci area all were recorded. The numbers of foci per unit volume of liver, the percent foci volume, mean focus area, and mean focus volume were calculated by the stereological procedures of Campbell et al. (1982).

#### *Enzyme Studies*

For the demonstration of ODC activity, a 20% homogenate of liver was prepared from a piece of liver removed from the right lobe of each animal. All enzyme activities were assayed using a cytosolic preparation (100,000  $\times$  g, 1 h) and normalized to protein content. ODC activity was determined using the procedure of Bethell and Pegg (1979). Protein was determined by the method of Bradford (1976).

The cyanide-insensitive peroxisomal  $\beta$ -oxidation of the palmitoyl coenzyme A (CoA) procedure of Lazarow (1982) was performed on a 1500  $\times$  g supernatant fraction of a 20% liver homogenate prepared in 0.25 M sucrose. The initial rate of oxidation was expressed as the amount of nicotinamide adenine dinucleotide formed per minute, and the rate was normalized either to protein content, gram of liver, or total liver. Protein was determined as outlined above.

#### *Statistics*

Body weights, liver-to-body weight ratios, and enzyme data were compared by means of the two-sample independent t-test ( $p < 0.05$ ). Foci and related parameters were compared by means of the one-factor Multivariable Analysis of Variance for Repeated Measures Test for missing data (Dixon et al., 1985).

## **RESULTS**

### ***Body and Liver Weight***

The body weight was not affected by the treatment. The liver-to-body weight ratio in the animals receiving PB as the tumor promoter were significantly higher than control (6.85% and 5.11%, respectively;  $p < 0.0005$ ).

### ***Enzyme Data***

A significant difference ( $p < 0.05$ ) in the activity of ODC between test and control groups ( $4.77 \pm 1.45$  and  $2.24 \pm 1.09$  pmol/0.5 h/mg protein, respectively) was observed. No differences were found in the activity of cyanide-insensitive palmitoyl CoA oxidation between test and control groups ( $1.75 \mu\text{mol}/\text{min/g}$  for both groups).

### ***Enzyme-Altered Foci***

No foci were detectable in liver sections stained with H&E or in sections stained by the PAS technique. Quantitation of foci from sections stained with the four other methods was accomplished and the results of stereological analyses are presented in Table 4.4-1. There were no significant differences in the number of foci detected by each staining procedure. Staining for the presence of G6Pase-deficient foci initially appeared to be the most sensitive marker of EAF in the current assay. However, the value obtained for the number of foci was misleading because one of the animals in the study had a high number of G6Pase-deficient foci. The mean of the foci per cubic centimeter from the other three animals was  $88.7 \pm 22.46$ . The value obtained for iron-deficient foci per cubic centimeter also was misleading because two animals had very high numbers of foci whereas two had much lower numbers of foci. Quantitation of ATPase-deficient foci yielded consistently lower numbers of foci than the other three methods, which may have been due to the very weak staining observed with this method. GGT-positive foci appeared to be the most sensitive marker for EAF in this study because the numbers of foci were consistently higher than those detected by other methods and the foci were easier to detect.

There were no significant differences in the volume of the liver occupied by foci (percent foci volume) detected by the four staining procedures. However, there were significant differences in the size of foci detected by each method. The foci that were positive for GGT were significantly smaller in area and volume than foci detected by iron staining, but they were not significantly different than foci detected by staining for G6Pase or ATPase. The foci detected by iron staining were nearly twice the size of GGT-positive foci and occupied nearly three times the area. The mean area and volume of the G6Pase-deficient foci was the same as the mean area and volume of ATPase-deficient foci, but were significantly smaller than iron-deficient foci.

TABLE 4.4-1. QUANTITATION<sup>a</sup> OF DEN-INITIATED ENZYME-ALTERED FOCI IN MALE SPRAGUE-DAWLEY RATS STAINED FOR GGT, ATPase, G6Pase, AND IRON.

STAIN	TREATMENT	N	FOCI/cm <sup>3</sup>	% FOCI VOL	MEAN FOCUS AREA (mm <sup>2</sup> )	MEAN FOCUS VOLUME (mm <sup>3</sup> )
GGT	DEN/PB	5	122.6 ± 15.23 <sup>b</sup>	0.075 ± 0.0167 <sup>b</sup>	0.025 ± 0.0053 <sup>b,c</sup>	0.007 ± 0.0020 <sup>b,c</sup>
	Control	5	19.3 ± 7.91	0.010 ± 0.0048	0.017 ± 0.0051	0.004 ± 0.0020
G6Pase	DEN/PB	4	101.7 ± 36.90 <sup>b</sup>	0.168 ± 0.0745 <sup>b</sup>	0.049 ± 0.0043 <sup>b</sup>	0.018 ± 0.0020 <sup>b</sup>
	Control	4	10.1 <sup>d</sup>	0.020 <sup>d</sup>	0.055 <sup>d</sup>	0.019 <sup>d</sup>
ATPase	DEN/PB	4	118.2 ± 56.40 <sup>b</sup>	0.143 ± 0.0930	0.027 ± 0.0040 <sup>c</sup>	0.008 ± 0.0018 <sup>c</sup>
	Control	4	n.d.	n.d.	n.d.	n.d.

<sup>a</sup> Values represent the mean of N determinations ± 1 SEM.

<sup>b</sup> Significantly different than control at p<0.01, as determined by the Multivariate Analysis of Covariance for Repeated Measures Test.

<sup>c</sup> Significantly different from iron group at p<0.01, as determined by the Multivariate Analysis of Covariance for Repeated Measures Test.

<sup>d</sup> Only one animal in the group had detectable foci.

n.d. = None detected.

## DISCUSSION

The most commonly used markers for EAF have included the deficiency of G6Pase activity, the deficiency of membrane-bound ATPase activity, increased GGT activity (Farber and Cameron, 1980; Bannasch, 1986), resistance to accumulation of iron (Williams, 1982), and increased glycogen storage (Bannasch, 1986). GGT staining has been the most widely used for quantitation of foci (Hanigan and Pitot, 1985; Hendrich and Pitot, 1987) because of the ease with which the foci are visualized and because 90 to 95% of the EAF produced in various rat liver experimental carcinogenesis models are GGT positive (Tatematsu et al., 1980; Goldfarb and Pugh, 1981). Iron-deficient foci have been shown to be nearly as consistent a marker as GGT (Williams, 1982). The least prevalent of the markers within EAF has been shown to be the loss of G6Pase (Hirota and Williams, 1979; Ogawa et al., 1980). Deficiency of this enzyme almost always has been associated with either increased GGT or a deficiency of ATPase (Ogawa et al., 1980). Recently, Sato et al. (1984) reported a remarkable increase in the enzyme GST-P in the liver of rats treated with hepatocarcinogens. Foci containing this enzyme have been demonstrated by immunohistochemical methods and a comparison between foci staining positive for both GST-P and GGT have revealed GST-P to be the most effective marker for DEN-initiated lesions. The antibody to GST-P has not been widely available and therefore staining for this marker in the present study could not be done.

The present study has shown that GGT-positive foci are produced in response to DEN initiation and that they are the best choice for the demonstration of foci produced in response to genotoxic

hepatocarcinogens, which is in agreement with those studies cited above. The numbers of GGT-positive foci per square centimeter produced in this study ( $2.9 \pm 0.36$ , data not shown) were similar to those reported by other investigators using a similar protocol (Tatematsu et al., 1979; Goldsworthy et al., 1984; Parnell, 1986). Iron-deficient foci also were shown to be a good marker in the present study. However, quantitation of foci was not consistent from animal to animal because two animals had foci that were comparable in number to those detected in liver sections stained for GGT-positive foci, whereas two had substantially lower numbers of foci. In the present study ATPase-deficient foci were found to be the least detectable, which is not in agreement with the findings of Hirota and Williams (1979) and Ogawa et al. (1980) who observed that G6Pase-deficient foci were the least prevalent marker for EAF. The difficulty in quantitation of ATPase-deficient foci may have been due, in part, to the storage of slides at  $-40^{\circ}\text{C}$  prior to staining. Although foci characterized by excessive storage of glycogen have been reported to be one of the earliest detectable markers (Bannasch et al., 1980), foci showing an increase in glycogen were not detectable in the present study. This may have been due to a masking of the glycogen by the counterstain.

The activities of two enzymes were evaluated in this study. The elevation of the enzyme ODC has served as a unique, early marker of the action of complete chemical carcinogens or tumor promoters (Olson and Russell, 1979, 1980). DEN administered as a single dose has been shown to cause elevation in ODC activity within hours but this elevation is not sustained unless a large dose of DEN is administered. DEN (200 mg/kg) resulted in a 8- to 14-fold elevation of liver ODC activity for only seven days following a single dose (Olson and Russell, 1979). Because the current study was conducted over a six-week period, an elevation of ODC in response to just the DEN was not expected. A long-term study of dietary PB as a promoter of DEN-induced hepatic carcinogenesis failed to demonstrate elevated ODC activity at any point in the study (Farwell et al., 1978) although it has been shown to stimulate ODC in other studies (Pereira et al., 1982). In the present study significant differences were found in the activity of ODC in the two groups of animals, but the difference in ODC activity, while statistically significant, was probably not biologically significant because much greater increases in ODC have been observed with tumor promoters.

The second enzyme activity examined in this study was the peroxisomal  $\beta$ -oxidation of palmitoyl CoA. Elevations of this enzyme activity have been correlated with an induction of peroxisomes (Stott, 1988). The chronic administration of chemicals causing peroxisomal proliferation has been associated with the development of malignant hepatic tumors (Reddy et al., 1980). Although DEN and PB have not been shown to be peroxisomal proliferators in this study and those of others (Cattley and Popp, 1989), the enzyme assay to detect this phenomenon was included in the study because future initiation/promotion studies will be conducted using compounds that may be peroxisomal proliferators. The ability to correlate the degree of enzyme induction with formation of

detectable foci will be essential to the understanding of the mechanism of tumor formation by peroxisome proliferators.

The results of this study have indicated that quantitation of EAF can be used to assess the carcinogenic potential of the standard genotoxic carcinogen, diethylnitrosamine. The procedures developed in this study currently are being used to assess the carcinogenic potential of CTFE trimer acid.

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SECTION 5  
AIR FORCE CHEMICAL DEFENSE AND CW AGENT TOXICITY

**5.1 DEVELOPMENT OF A PHYSIOLOGICALLY BASED MODEL FOR ORGANOPHOSPHATES:  
DETERMINATION OF *IN VITRO* TISSUE/BLOOD PARTITION COEFFICIENTS FOR PARAOXON**

J.M. Gearhart, S. Hsu, and M. Masnyk

**ABSTRACT**

The development of a physiologically based pharmacokinetic (PB-PK) model for organophosphates would be a useful tool to assist in the health risk assessment of Air Force personnel exposed to these toxic compounds. One of the major elements of the model development process is the determination of a toxic agent's solubility in different biological tissues. In this study an *in vitro* method was employed to determine the tissue solubility (partition coefficient) for the nonvolatile organophosphate paraoxon. *In vitro* tissue/blood partition coefficients were determined for paraoxon in male Fischer 344 (F-344) rats. There was good agreement between the values determined *in vitro* and the values that were calculated from data obtained from the literature.

**INTRODUCTION**

United States Air Force personnel may be exposed to organophosphorus (OP) nerve agents in combat situations resulting, for example, in diminished performance of pilots in high performance aircraft. There is also the potential for exposure of medical personnel who perform their duties in field hospitals. Because both of these situations would involve repeated, nonlethal exposures to OP agents, it is important to determine their effects on performance. Additionally, nonagent OPs occur as environmental contaminants and appropriate tools are needed for assessing the health risks associated with exposure to these toxicants.

Organophosphorus compounds are acutely toxic. They irreversibly inhibit acetylcholinesterase (AChE), causing an excess accumulation of the neurotransmitter acetylcholine at its receptor (Koelle, 1981). The resultant inhibition of normal nervous system function produces a broad spectrum of responses, including malfunction of secretory processes, cardiovascular effects (i.e., bradycardia), and paralysis of respiratory muscles, a significant element in OP-induced mortality. Central nervous system effects include confusion, ataxia, coma, and central respiratory system paralysis (Karczmar, 1984).

A PB-PK model describing the kinetics of OPs such as paraoxon and the inhibition of AChE in mammals would be of great value in predicting health risks associated with OP exposure. The goal of this work was to determine biochemical constants necessary for coding a PB-PK model for paraoxon

kinetics in the rat. Therefore, *in vitro* tissue/blood partition coefficients for paraoxon in male F-344 rats were determined.

## MATERIALS AND METHODS

### Test Material

Diethyl *p*-nitrophenyl phosphate (paraoxon) was obtained from Sigma Chemical Co. (St. Louis, MO). The test chemical was diluted with 0.1 M phosphate buffer before being used in the treatment of tissue homogenates or construction of standard curves.

### Animals

Male F-344 rats weighing 175 to 300 g were purchased from Charles River Breeding Labs, Kingston, NY. They were quarantined for a two-week period, during which in-house health assessments were conducted. Rats were group housed in clear plastic cages with wood-chip bedding. Food (Purina Formulab #5008) and water were available ad libitum. Animal room temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals.

### Quantitation of Paraoxon

Paraoxon was quantified in experimental samples by comparing inhibition of a standard AChE solution (electric eel, type III, Sigma, St. Louis, MO) to a standard curve in which known paraoxon concentrations were correlated with their respective degrees of AChE inhibition.

## IN VITRO TISSUE/BLOOD PARTITION COEFFICIENTS

### Heat/EDTA Inactivation

To determine the partitioning of this compound between tissue and saline it was necessary to first inactivate the esterases responsible for metabolism of paraoxon. Therefore, partition coefficients were determined after a scavenging of critical metal cofactors by ethylenediaminetetraacetic acid (EDTA) and heat inactivation of the tissue homogenates. Tissues were obtained at sacrifice (CO<sub>2</sub> inhalation), weighed, diluted 1:3 (wt/vol) with normal saline, and homogenized with a Tissumizer tissue homogenizer (Tekmar, Cincinnati, OH). A 80-mM solution of EDTA was added to each homogenate. Homogenates then were heat-treated at 60 °C for 1 h and allowed to cool to 37 °C in a heated vortex evaporator. Preliminary investigations had determined that the most significant inactivation of paraoxonase activity was accomplished after treatment with EDTA and 1 h of heat treatment at 60 °C. The degree of inactivation was determined by adding paraoxon to inactivated and control homogenates and measuring the production of *p*-nitrophenol spectrophotometrically. The absorbance of the treated homogenates was 0.01, whereas the untreated was 2.74.

### **Partition Coefficient Determination**

Tissue/saline partitions were determined for blood, lung, brain, liver, kidney, fat, muscle, and diaphragm. Blank vials containing normal saline were treated in parallel to serve as controls. Once the vials had reached 37 °C, they all were treated with paraoxon and incubated for 2 h. At the end of the 2 h incubation, 200 µL of incubate was withdrawn and placed in the sample reservoir of an ultrafiltration system (Amicon micropartition system, MPS-1, Amicon Corp., Danvers, MA). The samples were centrifuged at 2000 × g for 3 min and the paraoxon concentration in the supernatant of each tube was determined by the enzyme inhibition assay described in the quantification of paraoxon section. The tissue/blood partition coefficients were calculated as follows.

- Determination of the concentration of paraoxon in the saline-blank supernatant ( $T_s$ )
- Determination of the concentration of paraoxon in the tissue supernatant ( $T_t$ )
- Division of  $T_t/T_s$  to yield the tissue/saline partition

The tissue/blood partition coefficient then was obtained by dividing the tissue/saline coefficient by the blood/saline partition coefficient.

### **RESULTS**

Tissue/blood partition coefficients were determined *in vitro* for seven different tissues. The group mean partition coefficients are listed in Table 5.1-1.

**TABLE 5.1-1. IN VITRO TISSUE/BLOOD PARAOXON PARTITION COEFFICIENTS**

Tissue	Partition Coefficient	Number of Samples
Lung	1.87 (1.53) <sup>a</sup>	3 <sup>b</sup>
Brain	0.86 (0.22)	4
Liver	2.27 (2.38)	5
Kidney	1.15 (0.85)	3
Fat	1.54 (1.10)	5
Muscle	0.96 (0.32)	3
Diaphragm	0.80 (0.06)	3
Blood <sup>c</sup>	1.30 (0.23)	7

<sup>a</sup> Standard deviation of the mean

<sup>b</sup> Number of samples used to determine group mean.

<sup>c</sup> Blood/saline partition coefficient.

## **DISCUSSION**

A method for determining *in vitro* tissue/blood partition coefficients for nonvolatile chemicals has been developed and used for the organophosphate paraoxon. Measuring the values for this parameter is a critical step in the construction of a PB-PK model for any compound, because the partition coefficient is one of the main parameters that controls the concentration of a chemical in blood and tissues.

As an estimate of how well the *in vitro* method of determining partition coefficients used in this study predicts *in vivo* values, brain/blood and liver/blood partition coefficients were calculated from blood and tissue paraoxon concentrations measured by Eigenberg et al. (1983). In the Eigenberg study, rats were dosed intravenously with parathion, the parent compound of the metabolite paraoxon, and the concentrations of paraoxon were determined in blood, liver, and brain at different times over a 1-h period after injection. After the 1-h period, the tissue levels of paraoxon fell below the limit of detection of the analytical method. At that time point, the relative concentrations of paraoxon in liver and brain were compared to that in blood, as an estimate of *in vivo* partition coefficients for paraoxon. These *in vivo* brain/blood and liver/blood partition values were 1.04 and 2.39, respectively. The *in vitro* partitions determined here for these two tissues were 0.86 and 2.27, respectively. The range of *in vivo* partition was estimated by using plus or minus one standard deviation of the mean concentration of paraoxon for each tissue. This resulted in partition ranges for brain/blood of 0.71 to 1.14, and for the liver/blood of 2.29 to 2.44. Because the group mean concentrations and their standard deviations were used for this estimate, the actual variability of the *in vivo* partition values most likely are greater than those reported here. The *in vitro* method of determining tissue/blood partition coefficients for these two tissues provided a good estimate of the values calculated from the available *in vivo* data.

It should be noted that the *in vivo* tissue/blood partitions obtained from the Eigenberg study are only estimates of the actual tissue and blood solubilities of paraoxon. Metabolism of paraoxon would tend to decrease the tissue concentration below that expected on the basis of solubility. On the other hand, specific binding of paraoxon would increase its tissue concentration above that expected on the basis of solubility alone. Determination of the contribution of these processes, if any, to the *in vivo* partition coefficients estimated from the Eigenberg data is beyond the scope of this report. The general agreement of these partition coefficients with those determined *in vitro* suggests, however, that the tissue-specific metabolism and binding of paraoxon were not major determinants of the estimated partition coefficients.

Previous studies have used the concentration of chemicals in tissues versus blood after iv injection or constant rate infusion in the determination of partition coefficients (Chen and Gross; 1979, Gallo et al., 1987). The degree to which these methods allow the actual determination of a chemical's solubility in tissues versus blood is dependent on each chemical and the degree to which there is specific metabolism and binding of the chemical in different organs.

The *in vitro* determination of paraoxon tissue/blood partition coefficients provides part of the set of parameter measurements needed for a PB-PK model. Additional studies would be required for determination of other model parameters.

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## **5.2 DETERMINATION OF THE REPEATED INHALATION TOXICITY OF CHLOROPENTAFLUOROBENZENE**

**E.R. Kinkead, H.G. Wall, and R.S. Kutzman**

### **ABSTRACT**

This study was designed to evaluate the toxic effects associated with repeated or continuous exposure to chloropentafluorobenzene (CPFB) by both dermal and inhalation routes. Male and female Fischer 344 (F-344) rats were exposed for three weeks to air alone, 0.25, 0.50, or 1.00 mg CPFB/L. No deaths or signs of toxic stress occurred during the exposure period. A treatment-related depression in mean weight gain and increased numbers of pulmonary alveolar macrophages and renal hyaline droplets were noted in both sexes. Male and female New Zealand white (NZW) rabbits were treated dermally for three weeks with mineral oil, or 0.25, 0.50, or 1.00 g CPFB/kg. No toxic effects were noted in either sex of rabbits.

### **INTRODUCTION**

CPFB is a candidate material for use as a CW simulant for training purposes. Preliminary screening has indicated that CPFB provides good detectability for biological monitoring, desirable partitioning in biological tissues, acceptable physical properties, and relative biological inertness (Jepson et al., 1985).

The primary irritation hazard, sensitization potential, and acute inhalation toxicity of CPFB have been evaluated in this laboratory (Kinkead et al., 1987). CPFB demonstrated no potential for skin sensitization in guinea pigs; however, it did produce mild skin and conjunctival irritation in rabbits. Short-term exposure to CPFB vapor posed no serious hazard by the inhalation route: All rats survived a 4-h exposure of 4.84 mg/L. It is necessary to determine the effects of repeated inhalation exposure to this material because it is anticipated that, under the conditions of intended use, individuals may be exposed to this simulant on a short-term repeated or, in the case of instructors, recurring basis.

The rat was selected as the test species for the short-term repeated inhalation portion of this study. The species and numbers of rats per group were selected to conform with the U.S. Environmental Protection Agency's Health Effects Test Guidelines (1985) and to allow for significant statistical evaluation of the results. Existing alternative methods to animal testing are inadequate for this study.

## **METHODS AND EXPERIMENTAL EVALUATIONS**

A detailed description of the methods and experimental evaluations performed for this study was provided in the 1988 Toxic Hazards Research Unit Annual Report (Kinkead et al., 1989).

## **RESULTS**

A detailed description of the results of this study, with the exception of histopathology, was provided in the 1988 Toxic Hazards Research Unit Annual Report (Kinkead et al., 1989).

## **HISTOPATHOLOGY RESULTS**

Microscopic changes noted in F-344 rats that were attributable to exposure were limited to the livers of female rats and the kidneys of male rats (Table 5.2-1). Necrosis of individual hepatocytes was a consistent finding in the livers of CPFB-exposed female rats. Affected hepatocytes were characterized by pyknotic or karyorrhectic nuclei and increased cytoplasmic eosinophilia. In some instances necrotic hepatocytes were associated with variable numbers of mononuclear inflammatory cells, mostly macrophages and lymphocytes. In other areas, affected hepatocytes did not elicit such a cellular reaction. There was not a consistent pattern to the location of necrotic hepatocytes within the hepatic lobule. Although liver weights of CPFB-exposed male and female rats were elevated significantly at the conclusion of the study, there were no morphologic differences evident between control and test rats at the light microscopic level. The formation of hyaline droplets was a consistent finding in the proximal convoluted tubules of the kidneys of all CPFB-exposed male rats.

Microscopic lesions noted in B6C3F<sub>1</sub> mice that were attributable to exposure were limited to the liver (Table 5.2-2). In both male and female 2.50-mg CPFB/L-exposed mice, hepatocytes in the midzonal and, to a lesser degree, centrilobular regions of most hepatic lobules exhibited mild hepatocytomegaly characterized by increased amounts of finely granular eosinophilic cytoplasm. The enlarged hepatocytes tended to distort hepatic plate architecture with compression of sinusoidal spaces. The microscopic hepatocytomegaly correlates well (male mice  $r=0.58$ ,  $p<0.0028$ ; female mice  $r=0.89$ ,  $p<0.0001$ ) with the statistically significant ( $p<0.01$ ) increase in relative liver weights in the high-concentration group. Hepatocytomegaly and a corresponding increase in relative liver weight were not evident in the mid- or low-concentration CPFB-exposed groups.

Another treatment-related change in the livers of male and female mice was necrosis of individual hepatocytes. This lesion was present in all CPFB-exposed groups with approximately the same degree of severity. This lesion was similar morphologically to the liver changes described in female rats. Single-cell necrosis was evident in control group livers, but at a reduced incidence. Other microscopic changes noted in other organs were not treatment related, but were spontaneously occurring, age-related changes.

**TABLE 5.2-1. SUMMARY OF SELECTED MICROSCOPIC LESIONS OBSERVED IN F-344 RATS FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE**

Organ Lesion	Incidence (%)				Severity <sup>a</sup>			
	0.0 mg/L	0.25 mg/L	0.80 mg/L	2.50 mg/L	0.0 mg/L	0.25 mg/L	0.80 mg/L	2.50 mg/L
<b>Liver</b>								
Single-Cell Necrosis								
Male	0	0	0	0	0.0	0.0	0.0	0.0
Female	0	80b	90b	60b	0.0	0.8b	1.0b	0.6b
<b>Kidney</b>								
Tubular Mineralization								
Male	0	0	0	0	0.0	0.0	0.0	0.0
Female	50	0	0	90	0.5	0.0	0.0	0.9c
Hyaline Droplets								
Male	0	100b	100b	100b	0.0	1.0b	1.1b	1.5b
Female	0	0	0	0	0.0	0.0	0.0	0.0

<sup>a</sup> Severity scoring system defined as 0 = no lesion; 1 = minor or very slight; 2 = slight; 3 = moderate; 4 = marked; 5 = severe.

Group scores calculated by dividing the sum of individual scores by the number of affected animals.

b Significantly different from control, p<0.01, using Fisher's Exact Test and Yates' Corrected Chi-Square Test.

c Significantly different from control, p<0.05, using Fisher's Exact Test and Yates' Corrected Chi-Square Test.

**TABLE 5.2-2. SUMMARY OF SELECTED MICROSCOPIC LESIONS OBSERVED IN B6C3F<sub>1</sub> MICE FOLLOWING 21-DAY REPEATED INHALATION EXPOSURE TO CHLOROPENTAFLUOROBENZENE**

Organ Lesion	Incidence (%)				Severity <sup>a</sup>			
	0.0 mg/L	0.25 mg/L	0.80 mg/L	2.50 mg/L	0.0 mg/L	0.25 mg/L	0.80 mg/L	2.50 mg/L
<b>Liver</b>								
Single-Cell Necrosis								
Male	17	67b	83b	100b	0.2	0.7b	0.8b	1.0b
Female	50	100b	100b	83b	0.5	1.0b	1.0b	0.8b
Hepatocytomegaly								
Male	0	0	17	100b	0.0	0.0	0.2b	1.8b
Female	0	0	0	100b	0.0	0.0	0.0	2.0b

<sup>a</sup> Severity scoring system defined as 0 = no lesion; 1 = minor or very slight; 2 = slight; 3 = moderate; 4 = marked; 5 = severe.

Group scores calculated by dividing the sum of individual scores by the number of affected animals.

b Significantly different from control, p<0.01, using Fisher's Exact Test and Yates' Corrected Chi-Square Test.

## **DISCUSSION**

Of prime importance was the toxic effect of CPFB on the liver of mice exposed at the highest concentration. The morphologic results documented gross liver hypertrophy and microscopic hepatocytomegaly as the principal manifestations of CPFB-induced hepatotoxicity. The changes observed in alkaline phosphatase activity and liver weight ratios of exposed female rats were considered directly related to CPFB exposure because there was significant morphologic alteration in the hepatocytes. Although hyaline droplet accumulation in proximal tubules was prevalent in all exposed male rat kidneys, the severity of the lesion was minimal and renal epithelial necrosis or other morphometric indicators of renal disease were not present. The decrease in mean corpuscle volume (MCV) had no physiological significance because the red blood cells and the hematocrit used to compute MCV were within normal ranges. Although the blood urea nitrogen and total protein values of the high-concentration rats were different from controls, the differences were not considered physiologically significant because all values were within normal ranges reported by other investigators (Ringler and Dabich, 1979.) The basis for the decrease in aspartate aminotransferase (AST) values with increasing exposure concentrations has not been determined. Because AST is an enzyme that increases when there is muscle damage, liver cell injury, or reduced glomerular filtration, the changes in this enzyme are probably not indicative of a disease process.

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### **5.3 EFFECTS OF 90-DAY CHLOROPENTAFLUOROBENZENE INHALATION EXPOSURE OF FISCHER 344 RATS AND B6C3F<sub>1</sub> MICE**

**E.R. Kinkead, S.K. Bunger, and H.G. Wall**

#### **ABSTRACT**

Chloropentafluorobenzene (CPFB) has been identified as a candidate uptake simulant for non-persistent CW agents. Acute toxicity studies have shown that CPFB has limited adverse effects on laboratory animals. A 21-day inhalation study of rats and mice to 2.5, 0.8, and 0.25 mg CPFB/L resulted in reduced weight gain in the high-concentration male and female rats and identified the liver as a potential target organ. This multiconcentration inhalation study was designed to detect a no-observable-effect level associated with repeated exposure to CPFB. Male and female rats and mice were exposed to CPFB vapors for 13 weeks. No effects on body weight, clinical chemistries, or mortality were noted at the conclusion of the study. Histopathologic examination of tissues removed at sacrifice is incomplete.

#### **INTRODUCTION**

CPFB is a candidate material for use as a chemical warfare simulant by the military for training purposes. Preliminary screening has indicated that CPFB provides good detectability for biological monitoring, desirable partitioning in biological tissues, acceptable physical properties, and relative acute biological inertness (Jepson et al., 1985).

The primary irritation hazard, sensitization potential, and acute inhalation toxicity of CPFB have been evaluated in this laboratory (Kinkead et al., 1987). CPFB demonstrated no potential for skin sensitization in guinea pigs; however, it did produce mild skin and conjunctival irritation in rabbits. Short-term exposure to CPFB vapor posed no serious hazard by the inhalation route as all rats survived a 4-h exposure of 4.84 mg/L. The in-life portion of a 21-day inhalation study of CPFB at concentrations of 2.50, 0.80, and 0.25 mg/L was concluded recently at this laboratory (Kinkead et al., 1989). Mean body weights of male and female rats exposed to 2.50 mg CPFB/L were depressed significantly during the last two weeks of the three-week study. Clinical effects included significant increases in liver/body weight ratios in both sexes of rats and mice. The greatest increase over control values (71%) occurred in female mice. Histopathologic results of the 21-day inhalation study indicated significant hepatocytomegaly in both male and female mice exposed at 2.5 mg CPFB/L. Hepatocellular necrosis was seen in at least 67% of the test mice, but also was noted in 50% of the control mice. Hyaline droplets were noted in kidneys of all test male rats.

It is anticipated that under the conditions of intended use individuals may be exposed to this simulant on a short-term repeated or, in the case of instructors, recurring basis. Therefore, it is necessary to determine the effects of repeated inhalation exposures to this material.

This multiconcentration inhalation study was designed to detect a no-observable-effect level associated with repeated exposure to CPFB for 13 weeks. The study included mice, some of which were maintained for six-months postexposure to determine if treatment-related effects are reversible.

Both the rat and mouse have been selected as test species for the 13-week repeated inhalation study because of the effects seen following the 21-day inhalation study. The inhalation route was chosen because it is the most likely route of potential human exposure in the manufacture and use of CPFB. The species and numbers of animals per group were selected to conform with the Environmental Protection Agency Health Effects Guidelines (1985) and to allow for significant statistical evaluation of the results. Existing alternative methods to animal testing were inadequate for this study, which needs to investigate the effect of repeated treatment on intact mammalian systems.

#### **MATERIALS AND METHODS**

##### **Test Agent**

The CPFB used in this study was purchased from Aldrich Chemical Co., Milwaukee, WI. A Material Safety Data Sheet was not available from the manufacturer. The physical properties of CPFB are shown below.

Chemical Formula	C <sub>6</sub> ClF <sub>5</sub>
Molecular Weight	202
Boiling Point (°C)	117
Density (g/mL)	1.568
Vapor Pressure (mmHg, 25 °C)	14.1
CAS No.	344-07-0

##### **Test Agent Quality Control**

The purity of the test material was determined by capillary gas chromatography. A Varian 3700 gas chromatograph equipped with a thermal conductivity detector and a 50-m, 5% phenylmethyl silicone capillary column was used in conjunction with a Hewlett-Packard 3388 computing integrator to measure peak area and record chromatograms. Quality control profiles were made of the material as received and vaporized and of residue from the vaporization system.

##### **Animals**

Upon receipt from Charles River Breeding Labs (Kingston, NY), male and female Fischer 344 (F-344) rats, six weeks of age, and male and female B6C3F<sub>1</sub> mice, six weeks of age, were quality control tested and found to be in acceptable health. They were group housed (two to three per cage)

in clear plastic cages with wood-chip bedding prior to the study. The rats and mice (nine weeks of age at initial exposure) were individually housed and assigned to specific exposure cage locations during the study. The exposure cages were rotated daily in a clockwise manner (moving one position) within the 690-L inhalation chambers to compensate for any possible variation in chamber exposure conditions. Water and feed (Purina Formula #5008) were available ad libitum except during the inhalation exposure period when food was removed and when the animals were fasted for 10 h prior to sacrifice. Ambient temperatures were maintained at 21 to 25 °C and the light/dark cycle was set at 12-h intervals (light cycle starting at 0700 h).

#### ***Exposure Regimen and Response Assessment***

Eight male and eight female F-344 rats and 12 male and 12 female B6C3F<sub>1</sub> mice were placed in four 690-L inhalation chambers and exposed for 6 h/day, 5 days/week, for 13 weeks (65 exposures over a 90-day test period) to either air only or 10, 50, or 250 mg CPFB/m<sup>3</sup>. Records were maintained for body weights, signs of toxicity, and mortality. All rats and eight of the 12 mice per group were sacrificed following the final exposure. Four mice per group were maintained six months postexposure. Euthanasia was accomplished via halothane inhalation overdose. At sacrifice, gross pathology was performed and tissue was harvested for histopathologic examination. Wet tissue weights were determined on adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, and thymus. Tissues for histopathologic examination were fixed in 10% neutral-buffered formalin, trimmed, and further processed via routine methods for hematoxylin-eosin-stained paraffin-embedded sections (Luna, 1968).

Additionally, blood was drawn for hematology and clinical chemistry assays. Erythrocytes were enumerated on a Coulter counter (Coulter Electronics, Hialeah, FL), and sera for clinical chemistry evaluation were assayed on a Ektachem 700XR (Eastman Kodak, Rochester, NY). Selected hematological parameters and absolute leukocyte differentials were determined according to established procedures. Sera were processed according to the procedures in the Ektachem operations manual.

#### ***Statistical Analysis***

Comparisons of mean body weights were performed using the Multivariate Analysis of Covariance for Repeated Measures Test (Barcikowski, 1983; Dixon, 1985). A two-factorial analysis of variance with multivariate comparisons was used to analyze the hematology, clinical chemistry, and organ weight data.

#### **RESULTS**

A total of 64 F-344 rats and 96 B6C3F<sub>1</sub> mice were included in the 13-week inhalation study. There were no rat deaths resulting from exposure. One female mouse, exposed at 10 mg CPFB/m<sup>3</sup>,

was found dead following Exposure Day 29. Examination of this animal determined the cause of death to be leukemia, and not related to CPFB exposure.

No treatment-related effects on mean body weight gains were noted in either male or female rats or female mice during the 13-week study (Figures 5.3-1 and 5.3-2). The high-level male mouse group had a depression in mean body weights at the conclusion of the study; however, the difference was not statistically significant. Mice held postexposure have demonstrated a substantial weight gain during the postexposure period.

Blood chemistry data from these animals are listed in Tables 5.3-1 through 5.3-4. Alkaline phosphatase values were significantly ( $p < 0.01$ ) increased in both male and female mice exposed to 250 mg CPFB/m<sup>3</sup>. Male mice exposed at 50 mg CPFB/m<sup>3</sup> also had an increase in this enzyme. Male rats from the 250-mg CPFB/m<sup>3</sup> exposure group had mean chloride levels significantly ( $p < 0.01$ ) elevated above values for other groups. All other parameters were within normal limits.

No concentration-related differences in relative organ weights occurred in either rats or mice exposed to CPFB (Tables 5.3-5 through 5.3-8). An increase in relative liver weight of the 50 mg CPFB/m<sup>3</sup> male mouse group was noted; however, no increase was noted in the relative liver weights of male mice exposed at five times that concentration. The significance of the relative weight difference will have to be determined through histopathologic examination.

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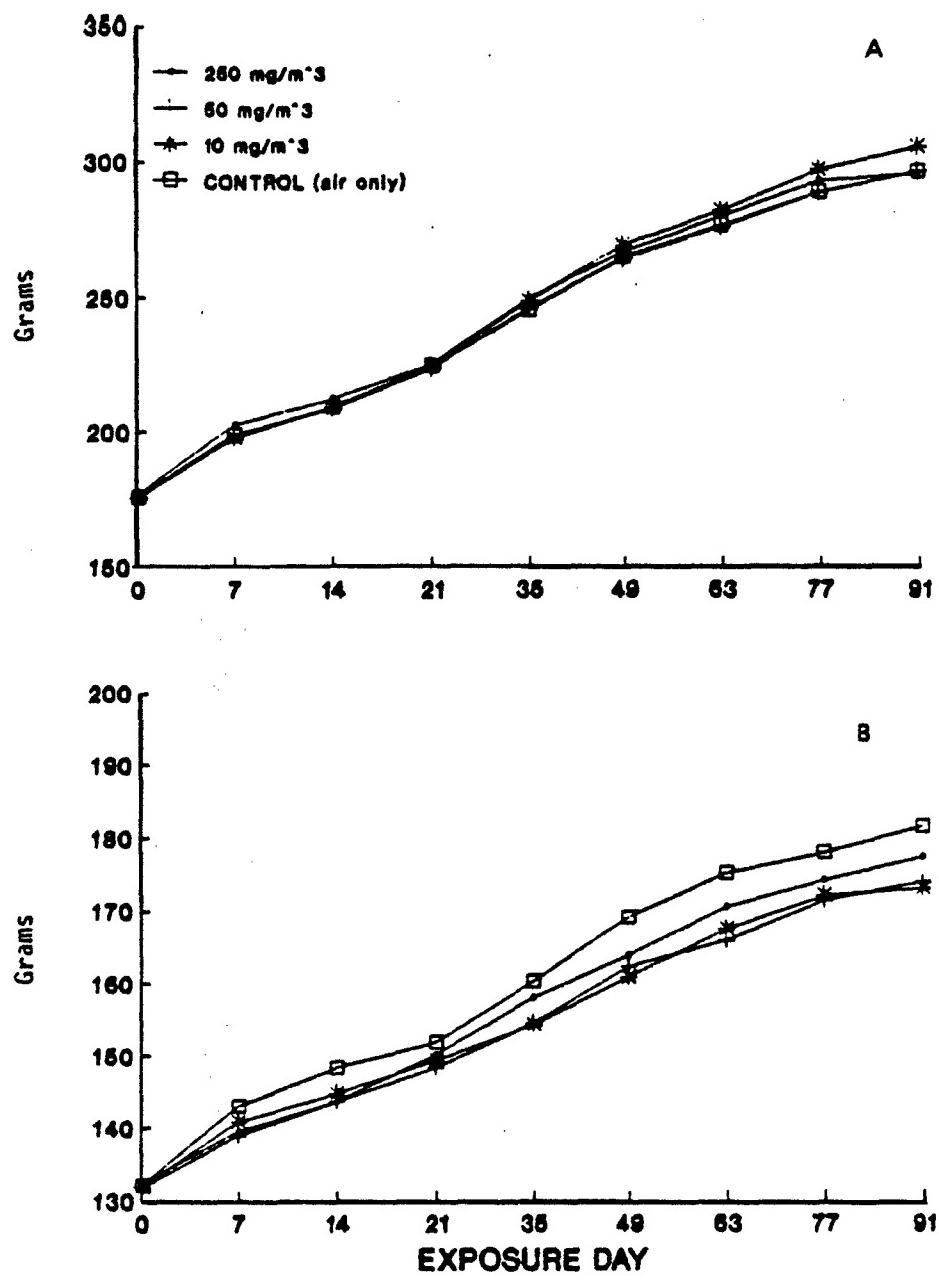


Figure 5.3-1. Mean Body Weights of Male (A) and Female (B) Rats Exposed to CPFB Vapors for 13 Weeks.

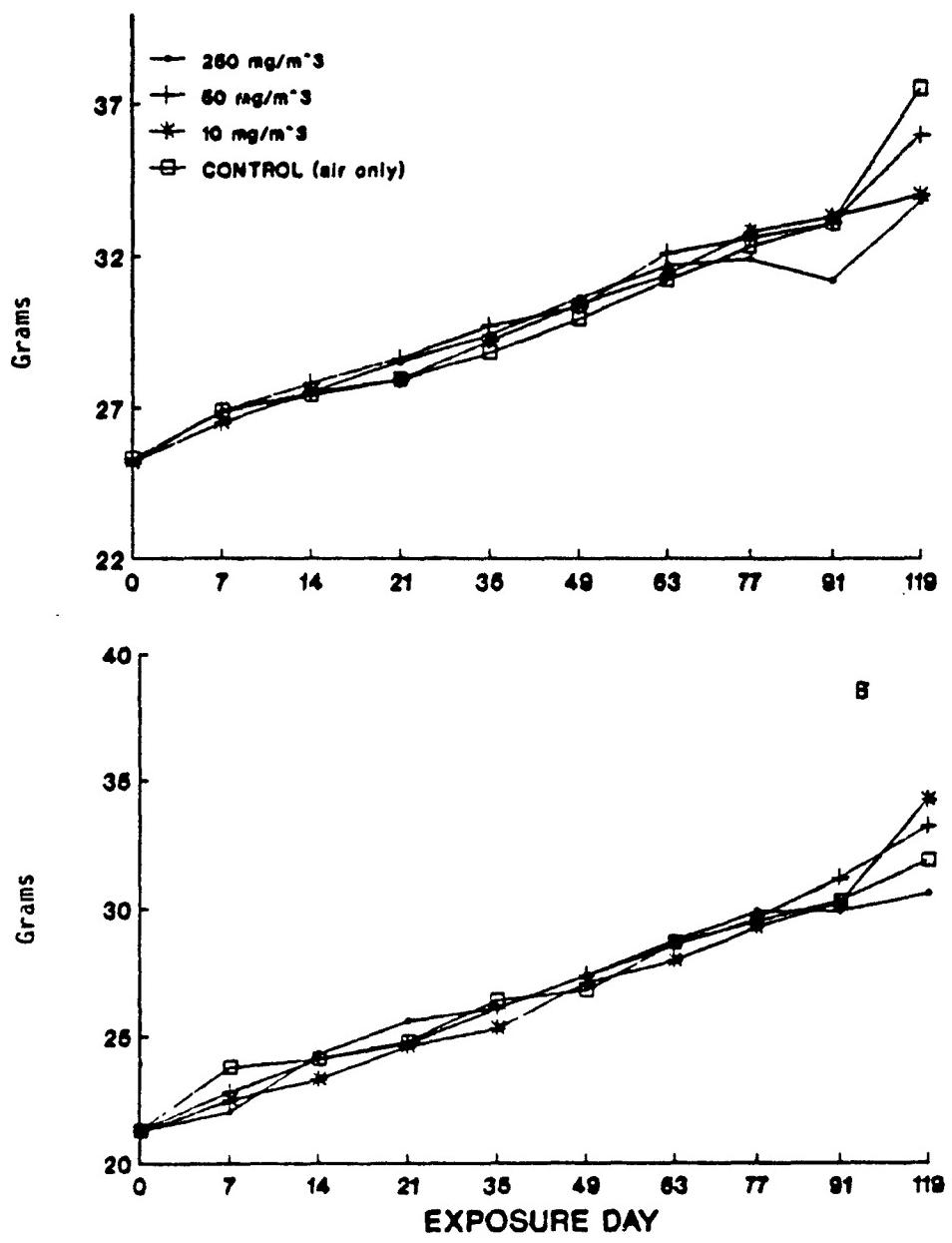


Figure 5.3-2. Mean Body Weights of Male (A) and Female (B) Mice Exposed to CPFB Vapors for 13 Weeks.

TABLE 5.3-1. MEAN<sup>a</sup> SERUM CHEMISTRY PARAMETERS FOR MALE F-344 RATS FOLLOWING 90-DAY REPEATED INHALATION EXPOSURE TO CPFB

Parameter	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Glucose (mg/dL)	128.6 ± 6.2	124.4 ± 6.0	124.4 ± 4.8	122.3 ± 6.6
Urea nitrogen (mg/dL)	12.0 ± 0.5	12.2 ± 0.7	13.2 ± 0.2	12.7 ± 0.5
Creatinine (mg/dL)	0.3 ± <0.1	0.4 ± <0.1	0.3 ± <0.1	0.4 ± <0.1
Sodium (mmol/L)	145.0 ± 0.3	145.4 ± 0.2	145.8 ± 0.4	145.6 ± 0.5
Potassium (mmol/L)	5.2 ± 0.1	5.0 ± 0.2	5.1 ± 0.1	5.1 ± 0.1
Chloride (mmol/L)	101.1 ± 0.7	101.0 ± 0.7	102.5 ± 0.6	128.9 ± 8.2 <sup>b</sup>
Calcium (mg/dL)	10.7 ± 0.1	10.8 ± 0.1	10.8 ± 0.1	10.9 ± 0.1
Phosphorus (mg/dL)	8.3 ± 0.2	7.8 ± 0.2	8.3 ± <0.1	8.6 ± 0.2
Cholesterol (mg/dL)	45.0 ± <0.1	45.1 ± <0.1	45.0 ± <0.1	46.3 ± 1.3
Total protein (g/dL)	6.1 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.4 ± 0.1
Albumin (g/dL)	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± <0.1
SGOT/AST (IU/L)	127.0 ± 8.9	124.6 ± 10.7	129.1 ± 7.3	120.9 ± 6.0
SGPT/ALT (IU/L)	67.5 ± 3.6	63.4 ± 2.6	67.4 ± 2.6	57.6 ± 2.4
Alkaline phosphatase (IU/L)	108.0 ± 3.1	103.0 ± 4.3	104.9 ± 13.7	108.8 ± 3.6

<sup>a</sup> Mean ± SEM, N = 8.

<sup>b</sup> Significantly different from controls at p < 0.01, as determined by a two-factorial analysis of variance with multivariate comparisons.

TABLE 5.3-2. MEAN<sup>a</sup> SERUM CHEMISTRY PARAMETERS FOR FEMALE F-344 RATS FOLLOWING 90-DAY REPEATED INHALATION EXPOSURE TO CPF B

Parameter	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Glucose (mg/dL)	109.9 ± 5.0	95.5 ± 4.5	105.4 ± 2.7	105.0 ± 6.4
Urea nitrogen (mg/dL)	15.4 ± 0.8	14.6 ± 0.5	13.9 ± 0.7	16.1 ± 0.9
Creatinine (mg/dL)	0.4 ± <0.1	0.4 ± <0.1	0.3 ± <0.1	0.4 ± <0.1
Sodium (mmol/L)	144.5 ± 0.3	143.4 ± 0.5	144.9 ± 0.3	143.8 ± 0.4
Potassium (mmol/L)	5.1 ± 0.1	5.3 ± 0.2	5.0 ± 0.1	5.0 ± 0.1
Chloride (mmol/L)	101.7 ± 0.1	100.2 ± 0.5	100.6 ± 0.6	100.3 ± 0.5
Calcium (mg/dL)	10.9 ± 0.1	11.0 ± 0.1	10.9 ± 0.1	11.0 ± 0.1
Phosphorus (mg/dL)	7.7 ± 0.4	8.2 ± 0.3	7.9 ± 0.3	7.2 ± 0.3
Total protein (g/dL)	6.4 ± 0.2	6.3 ± 0.1	6.3 ± 0.1	6.5 ± 0.1
Albumin (g/dL)	3.5 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.6 ± 0.1
SGOT/AST (IU/L)	110.0 ± 5.6	103.6 ± 6.6	99.4 ± 3.9	101.3 ± 3.9
SGPT/ALT (IU/L)	56.5 ± 4.3	50.4 ± 2.8	50.1 ± 1.3	50.8 ± 2.1
Alkaline phosphatase (IU/L)	89.8 ± 6.8	85.6 ± 4.1	89.4 ± 3.0	80.0 ± 4.3

<sup>a</sup> Mean ± SEM, N = 8.

**TABLE 5.3-3. MEAN<sup>a</sup> SERUM CHEMISTRY PARAMETERS FOR MALE B6C3F<sub>1</sub> MICE FOLLOWING 90-DAY REPEATED INHALATION EXPOSURE TO CPFB**

Parameter	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Urea nitrogen (mg/dL)	28.0 ± 14.1	16.3 ± 1.4	17.8 ± 1.8	13.5 ± 1.5
Creatinine (mg/dL)	0.1 ± <0.1	0.1 ± <0.1	0.1 ± <0.1	0.1 ± 0.1
SGOT/AST (IU/L)	337.9 ± 96.2	195.9 ± 84.2	278.9 ± 53.5	366.0 ± 119.8
SGPT/ALT (IU/L)	342.0 ± 88.6	118.9 ± 60.7	378.6 ± 111.1	456.4 ± 164.1
Alkaline phosphatase (IU/L)	79.8 ± 6.1	82.7 ± 8.2	109.5 ± 4.3 <sup>b</sup>	96.9 ± 3.2 <sup>b</sup>

<sup>a</sup> Mean ± SEM.

<sup>b</sup> Significantly different from controls at p<0.01, as determined by a two-factorial analysis of variance with multivariate comparisons.

**TABLE 5.3-4. MEAN<sup>a</sup> SERUM CHEMISTRY PARAMETERS FOR FEMALE B6C3F<sub>1</sub> MICE FOLLOWING 90-DAY REPEATED INHALATION EXPOSURE TO CPFB**

Parameter	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Urea nitrogen (mg/dL)	16.5 ± 1.1	15.7 ± 1.0	21.5 ± 4.9	16.1 ± 1.8
Creatinine (mg/dL)	0.1 ± <0.1	0.1 ± <0.1	0.1 ± 0.1	0.1 ± 0.1
SGOT/AST (IU/L)	82.6 ± 12.9	99.3 ± 17.5	85.0 ± 9.3	84.8 ± 17.4
SGPT/ALT (IU/L)	28.1 ± 3.5	33.3 ± 3.6	30.6 ± 3.8	22.8 ± 6.4
Alkaline phosphatase (IU/L)	118.6 ± 7.8	127.2 ± 4.4 <sup>b</sup>	121.3 ± 5.0	147.8 ± 8.2

<sup>a</sup> Mean ± SEM.

<sup>b</sup> Significantly different from controls at p<0.01, as determined by a two-factorial analysis of variance with multivariate comparisons, with multivariate comparisons.

**TABLE 5.3-5. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CPFB**

Organ	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Kidney Ratio	1.81 ± 0.05 0.64 ± 0.01	1.87 ± 0.03 0.64 ± 0.01	1.87 ± 0.05 0.71 ± 0.05	1.89 ± 0.02 0.67 ± <0.01
Heart Ratio	0.91 ± 0.01 0.32 ± 0.01	0.92 ± 0.02 0.31 ± 0.01	0.88 ± 0.03 0.34 ± 0.03	0.89 ± 0.02 0.32 ± 0.01
Brain Ratio	1.86 ± 0.06 0.66 ± 0.03	1.80 ± 0.02 0.62 ± 0.02	1.78 ± 0.03 0.68 ± 0.06	1.75 ± 0.0 0.62 ± 0.01
Liver Ratio	7.19 ± 0.23 2.52 ± 0.02	7.56 ± 0.23 2.58 ± 0.03	7.51 ± 0.25 2.84 ± 0.19	7.69 ± 0.14 2.74 ± 0.03
Spleen Ratio	0.58 ± 0.02 0.20 ± <0.01	0.60 ± 0.01 0.21 ± 0.01	0.58 ± 0.01 0.22 ± 0.02	0.56 ± <0.01 0.20 ± <0.01
Thymus Ratio	0.31 ± 0.03 0.11 ± 0.01	0.27 ± 0.01 0.09 ± <0.01	0.31 ± 0.02 0.12 ± 0.01	0.28 ± 0.02 0.10 ± 0.01
Lungs Ratio	1.62 ± 0.05 0.57 ± 0.02	1.77 ± 0.06 0.61 ± 0.02	1.65 ± 0.06 0.63 ± 0.04	1.61 ± 0.06 0.57 ± 0.02
Adrenals Ratio	0.04 ± <0.01 0.02 ± <0.01	0.05 ± <0.01 0.02 ± <0.01	0.05 ± <0.01 0.02 ± <0.01	0.05 ± <0.01 0.02 ± <0.01
Testes Ratio	2.97 ± 0.05 1.05 ± 0.02	2.92 ± 0.04 1.00 ± 0.01	2.92 ± 0.04 1.11 ± 0.10	2.91 ± 0.04 1.04 ± 0.02
Whole Body	285.1 ± 7.7	292.7 ± 7.1	272.8 ± 18.1	281.2 ± 3.0

<sup>a</sup> Mean ± SEM, N = 8.

**TABLE 5.3-6. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE F-344 RATS FOLLOWING 90-DAY INHALATION EXPOSURE TO CPFB**

Organ	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Kidney	1.18 ± 0.02	1.14 ± 0.01	1.17 ± 0.02	1.17 ± 0.02
Ratio	0.68 ± 0.01	0.69 ± 0.01	0.70 ± 0.01	0.69 ± 0.02
Heart	0.64 ± 0.01	0.59 ± 0.01	0.64 ± 0.02	0.62 ± 0.01
Ratio	0.37 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.37 ± 0.01
Brain	1.74 ± 0.02	1.75 ± 0.01	1.72 ± 0.02	1.72 ± 0.03
Ratio	1.01 ± 0.01	1.06 ± 0.02	1.03 ± 0.02	1.01 ± 0.01
Liver	4.61 ± 0.12	4.41 ± 0.11	4.44 ± 0.12	4.69 ± 0.05
Ratio	2.67 ± 0.05	2.66 ± 0.04	2.65 ± 0.02	2.76 ± 0.04
Spleen	0.43 ± 0.01	0.42 ± 0.01	0.41 ± 0.02	0.40 ± 0.01
Ratio	0.25 ± 0.01	0.25 ± 0.0	0.24 ± 0.01	0.24 ± <0.01
Thymus	0.23 ± 0.01	0.21 ± 0.01	0.22 ± 0.25	0.23 ± 0.01
Ratio	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
Lungs	1.20 ± 0.04	1.30 ± 0.03	1.27 ± 0.02	1.30 ± 0.04 <sup>b</sup>
Ratio	0.69 ± 0.02	0.79 ± 0.02	0.76 ± 0.02	0.76 ± 0.02 <sup>b</sup>
Adrenals	0.06 ± <0.01	0.05 ± <0.01	0.06 ± <0.01	0.05 ± <0.01
Ratio	0.03 ± <0.01	0.03 ± <0.01	0.03 ± <0.01	0.03 ± <0.01
Ovaries	0.11 ± 0.01	0.10 ± <0.01	0.10 ± 0.01	0.10 ± <0.01
Ratio	0.07 ± 0.01	0.06 ± <0.01	0.06 ± <0.01	0.06 ± <0.01
Whole Body	172.9 ± 3.1	165.9 ± 2.7	167.7 ± 4.3	169.9 ± 3.0

<sup>a</sup> Mean ± SEM, N = 8.  
<sup>b</sup> N = 7.

TABLE 5.3-7. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF MALE B6C3F<sub>1</sub> MICE FOLLOWING 90-DAY INHALATION EXPOSURE TO CPFB

Organ	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Kidney Ratio	0.57 ± 0.01 1.91 ± 0.06	0.61 ± 0.01 1.92 ± 0.04	0.56 ± 0.01 1.88 ± 0.04	0.56 ± 0.02 1.86 ± 0.04
Heart Ratio	0.16 ± <0.01 0.54 ± 0.02	0.18 ± <0.01 <sup>c</sup> 0.57 ± 0.02	0.16 ± <0.01 0.54 ± 0.01	0.17 ± <0.01 0.56 ± 0.01
Brain Ratio	0.44 ± 0.01 1.48 ± 0.06	0.45 ± 0.01 1.53 ± 0.04	0.44 ± 0.01 1.48 ± 0.05	0.45 ± 0.01 1.42 ± 0.05
Liver Ratio	1.32 ± 0.03 4.40 ± 0.0	1.46 ± 0.05 4.57 ± 0.19	1.96 ± 0.04 <sup>c</sup> 6.57 ± 0.15 <sup>c</sup>	1.38 ± 0.03 4.67 ± 0.11
Spleen Ratio	0.07 ± 0.01 0.23 ± 0.02	0.08 ± <0.01 0.24 ± 0.02	0.07 ± <0.01 0.25 ± 0.01	0.07 ± <0.01 0.24 ± 0.02
Thymus Ratio	0.04 ± <0.01 0.12 ± 0.01	0.03 ± <0.01 0.11 ± 0.01	0.03 ± <0.01 0.11 ± 0.01	0.04 ± <0.01 0.13 ± 0.01
Lungs Ratio	0.28 ± 0.01 0.95 ± 0.05	0.30 ± 0.01 0.94 ± 0.04	0.28 ± 0.01 0.94 ± 0.05	0.28 ± 0.01 0.96 ± 0.03
Adrenals Ratio	0.01 ± <0.01 0.03 ± <0.01	0.01 ± <0.01 0.04 ± <0.01	0.01 ± <0.01 0.03 ± <0.01	0.01 ± <0.01 0.03 ± 0.01
Testes Ratio	0.23 ± 0.01 0.77 ± 0.03	0.22 ± 0.02 0.69 ± 0.09	0.24 ± <0.01 0.81 ± 0.03	0.24 ± 0.01 0.83 ± 0.03
Whole Body	30.0 ± 0.8	31.8 ± 0.5	29.8 ± 0.7	29.2 ± 0.5

<sup>a</sup> Mean ± SEM, N = 8.

<sup>b</sup> N = 6.

<sup>c</sup> Significantly different from controls at p < 0.01 level, as determined by a two-factorial analysis of variance with multivariate comparisons.

**TABLE 5.3-8. ORGAN WEIGHTS (g)<sup>a</sup> AND ORGAN-TO-BODY WEIGHT RATIOS (%) OF FEMALE B6C3F<sub>1</sub> MICE FOLLOWING 90-DAY INHALATION EXPOSURE TO CPFB**

Organ	Control	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
Kidney Ratio	0.44 ± 0.02 1.53 ± 0.05	0.42 ± 0.01 1.53 ± 0.06	0.41 ± 0.01 1.48 ± 0.05	0.41 ± 0.02 1.51 ± 0.07
Heart Ratio	0.15 ± <0.01 0.54 ± 0.02	0.15 ± <0.01 0.54 ± 0.02	0.15 ± <0.01 0.54 ± 0.02	0.16 ± 0.01 <sup>c</sup> 0.59 ± 0.02
Brain Ratio	0.50 ± 0.01 1.76 ± 0.05	0.48 ± 0.01 1.75 ± 0.09	0.48 ± 0.01 1.75 ± 0.08	0.48 ± 0.01 1.76 ± 0.08
Liver Ratio	1.28 ± 0.03 4.51 ± 0.10	1.28 ± 0.06 4.61 ± 0.12	1.32 ± 0.05 4.79 ± 0.15	1.34 ± 0.05 4.91 ± 0.16
Spleen Ratio	0.10 ± 0.01 0.34 ± 0.02	0.10 ± <0.01 0.35 ± 0.02	0.09 ± <0.01 0.34 ± 0.02	0.10 ± 0.01 0.37 ± 0.02
Thymus Ratio	0.05 ± <0.01 0.18 ± 0.01	0.05 ± <0.01 0.19 ± 0.02	0.05 ± <0.01 0.19 ± 0.02	0.06 ± <0.01 0.20 ± 0.02
Lungs Ratio	0.27 ± 0.01 0.94 ± 0.03	0.28 ± 0.01 1.03 ± 0.04	0.27 ± 0.01 1.00 ± 0.03	0.28 ± 0.01 1.03 ± 0.04
Adrenals Ratio	0.02 ± <0.01 0.05 ± 0.01	0.01 ± <0.01 0.05 ± 0.01	0.01 ± <0.01 0.05 ± 0.01	0.01 ± <0.01 0.05 ± 0.01
Ovaries Ratio	0.03 ± <0.01 0.10 ± 0.01	0.03 ± <0.01 0.10 ± 0.01	0.03 ± <0.01 0.09 ± 0.01	0.02 ± <0.01 0.08 ± 0.01
Whole Body	28.5 ± 0.5	27.7 ± 0.9	27.7 ± 0.8	27.4 ± 0.8

<sup>a</sup> Mean ± SEM, N = 8.

<sup>b</sup> N = 7.

<sup>c</sup> Significantly different from controls at p < 0.01 level, as determined by a two-factorial analysis of variance with multivariate comparisons.

## **5.4 SUBCHRONIC INHALATION TOXICITY OF CHLOROPENTAFLUOROBENZENE ATMOSPHERE GENERATION AND CHARACTERIZATION**

**C.R. Doarn and D.A. Mahle**

### **ABSTRACT**

Chloropentafluorobenzene (CPFB), a candidate chemical warfare simulant, was recently studied to determine its subchronic inhalation toxicity. The exposures were conducted in the THRU inhalation chambers for a period of 90 days for 6 h per day, 5 days per week. The rat and mouse were exposed to chamber atmospheres containing 251.0, 50.4, and 10.1 mg CPFB vapor/m<sup>3</sup> of air, as determined by gas chromatography.

### **INTRODUCTION**

CPFB, a candidate material for use as a CW simulant and detectable in biological tissue, is under consideration by the Air Force for military training purposes. To determine the inhalation toxicity of this material, a multiconcentration subchronic inhalation study was conducted to define a no-observable-effect level. CPFB was generated as a homogeneous atmosphere by vaporization and was quantitated by gas chromatography (GC).

### **MATERIALS AND METHODS**

#### **Generation Method**

CPFB has a vapor pressure that is sufficient to allow it to be completely vaporized. To achieve a saturated atmosphere of CPFB vapor, a generator consisting of a gas-washing bottle containing CPFB and a fritted glass bubbler was constructed. To increase the surface area for vaporization, air was metered through the glass frit (Figure 5.4-1) with a rotameter (Matheson, Secaucus, NJ). The test atmosphere in the low (10.1 mg CPFB vapor/m<sup>3</sup>) chamber was generated using a midget impinger. Irrespective of the generation technique, the vaporized CPFB was transported through a 1/8 in.-stainless steel line into the exposure chamber.

To increase mixing efficiency, the flow of contaminant was introduced countercurrent to chamber input air flow. Mixing also occurred as the vapor exited the cone of the chamber through an adjustable annular orifice into the main body of the exposure chamber.

The exposures were conducted in Hinnens-type inhalation chambers for 90 days, 6 h/day, 5 days/week. Consumption rates for the generators used in the 250- and 50-mg/m<sup>3</sup> levels were approximately 27.4 and 8.7 g/day, respectively. These generators operated at 60% efficiency for the 250-mg/m<sup>3</sup> level and 37% efficiency for the 50-mg/m<sup>3</sup> level. The generator reservoir for the 10-mg/m<sup>3</sup> level was filled with fresh CPFB at the beginning of each week and was maintained during the week.

The atmosphere within the inhalation chamber was maintained by adjusting the chamber air flow, the level of CPFB in the reservoir, and the flow rate of air through the frit. The level of CPFB in the reservoir was maintained by using a peristaltic pump. The chambers were operated under a slight negative pressure (-1.5 in. of water) and the volume of air in the chamber was changed 12 to 15 times per hour.

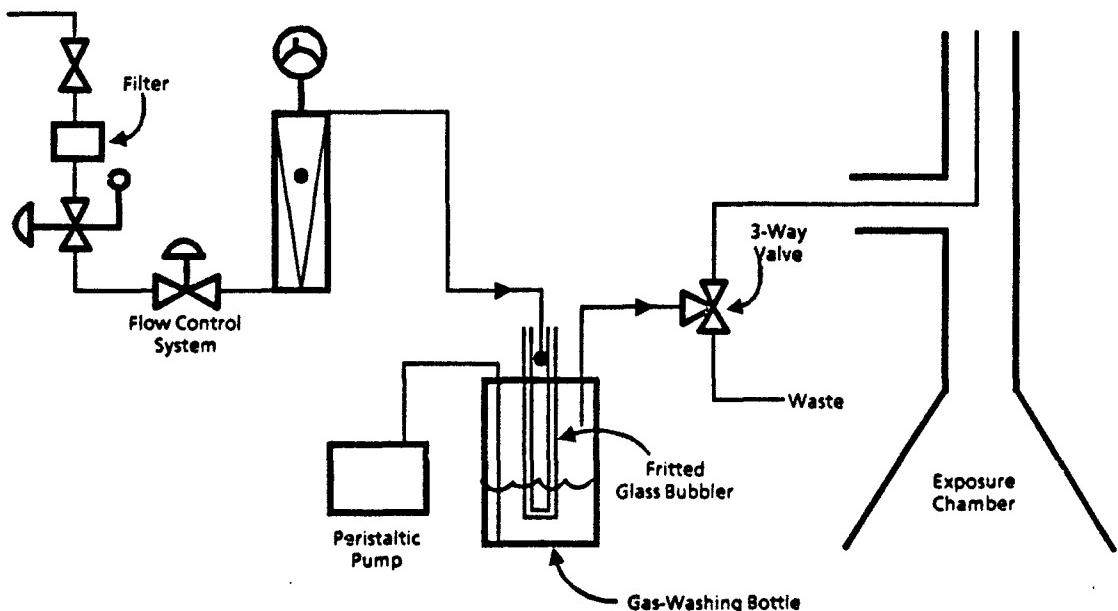
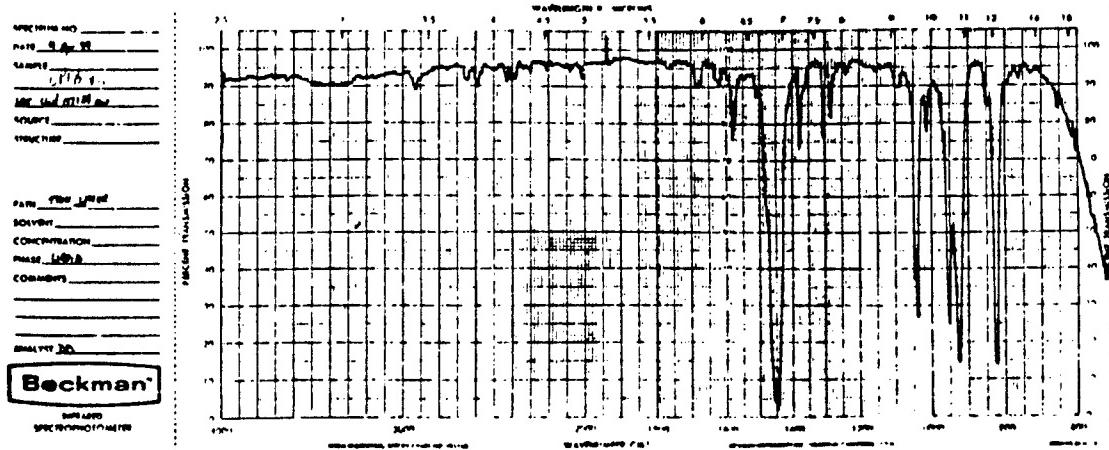


Figure 5.4-1. Schematic Diagram of the Experimental Apparatus.

#### Analytical Chemistry

Four separate lots of CPFB were received from Aldrich Chemical (Milwaukee, WI). Quality control analyses were performed on the stock material. Samples removed from each container were analyzed on a Beckman infrared spectrophotometer using NaCl plates. Figure 5.4-2 is a representative example of the infrared spectrum of CPFB. Comparison of the spectrum obtained from each lot indicated no significant difference between the samples of stock material.

Previous work performed at this laboratory with CPFB indicated that chamber concentration could easily be determined using infrared spectroscopy. During developmental work it was shown that the major band of absorptivity, characteristic of CPFB, was approximately 6.7  $\mu\text{m}$ . This wavelength would yield the greatest sensitivity when the test material was present in small quantities in the exposure chamber.



**Figure 5.4-2. Infrared Spectrum of Chloropentafluorobenzene.**

The problem associated with using infrared spectroscopy for this particular analysis was the absorption of water at a wavelength in the range of 5.5 to 5.8  $\mu\text{m}$ . This rather wide water band was shown to encroach on the band for CPFB. Although extra steps could be taken to minimize the presence of water, a full complement of animals in the chamber would result in an increase in water vapor in the chamber atmosphere. The interference would be visible, especially in the 10-mg/m<sup>3</sup> level.

To eliminate this problem a second method was developed to quantitate CPFB in the exposure atmosphere. This method, a GC approach, was employed as the primary determination of chamber concentration. The infrared analyzers were used to monitor the chambers continuously, thus providing the operator with a view of what was taking place in the exposure chamber in real time.

The Varian 1200 GC was operated under the following parameters. All data were integrated and reported by a Hewlett-Packard 3392A integrator.

#### Gas Chromatography Conditions

Column:	SE 30 10% 80/100 Chrom W
Length:	11 ft. x 1/8 in. stainless steel
Column temp:	170 °C
Injector temp:	250 °C, isothermal, flame ionization
Run time:	4.25 min
Sample loop:	1 mL
Carrier gas:	He @ 20 psi, H <sub>2</sub> @ 15 psi, air @ 20 psi
Sample flow rate:	7.0 Lpm

Hewlett-Packard Integrator, Model 3392A, SN 2408A03417

The GC, located directly behind the chambers to minimize transit time and possible adsorption of the material in the sampling line, was connected to each chamber through a computer-controlled, automatic-valving system. Figure 5.4-3 is a schematic of the sampling system.

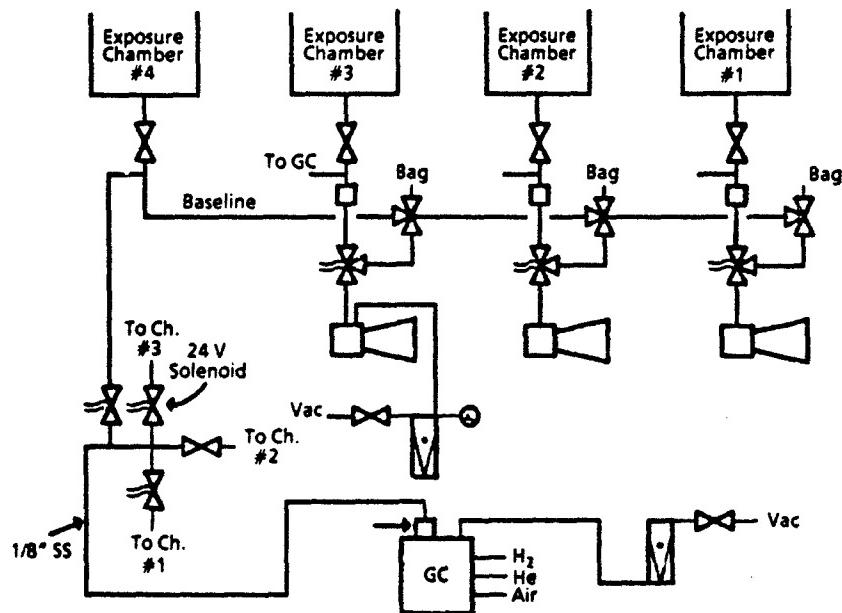


Figure 5.4-3. Schematic of Analytical System.

Samples were taken every 5 min, cycling from the control chamber to the 250-mg/m<sup>3</sup> level chamber at 20-min intervals. Peak areas were correlated with standard bag concentrations to achieve a standard linear curve using the least squares method. Figure 5.4-4, a chromatogram of CPFB, indicates the peak of interest at a retention time of approximately 2 min. The first peak represents some constituents present in the air. This peak also appears in a blank taken from the control chamber and does not interfere with the peak of interest.

During the normal operation of the analytical system for this study, four separate calibration curves were developed. These calibration curves, displayed in Table 5.4-1, were created because of the changes in the performance of the GC after an H<sub>2</sub> tank and three air tanks were changed. After these tanks were changed a calibration check was performed at two different levels on the standard curve. If the response from the instrument was within 10% of the target concentration, the instrument was deemed to be functioning properly. If, however, these calibration checks deviated from the 10% range, the instrument was recalibrated. Twice a week the calibration of the instrument was checked with standard bags to ensure good instrument performance.



RUN # 47                    JUL/13/89 11:48:36      RUN # 46                    JUL/13/89 11:43:56  
 WORKFILE ID: 8              WORKFILE ID: 8  
 WORKFILE NAME:             WORKFILE NAME:  
 AREA%                        AREA%  
 RT            AREA TYPE    AR/HT      AREA%                    RT            AREA TYPE    AR/HT      AREA%  
 1.27        2333          PB          0.141      42.57%        1.27        2642          BB          0.153      73.29%  
 2.19        3142          PB          0.131      57.42%        2.62        967          PY          0.291      26.71%

TOTAL AREA= 5488            TOTAL AREA= 3609  
 MUL FACTOR= 1.0000E+00    MUL FACTOR= 1.0000E+00

Figure 5.4-4. Chromatograms of CPFB and Blank.

TABLE 5.4-1. STANDARD CURVES FROM THE LEAST SQUARES METHOD

Date	Slope	Y-Intercept
5-02-89	256.15381	277.493694
5-22-89	172.76489	243.015226
6-12-89	201.33806	160.897082
7-12-89	292.27138	515.961458

#### Error Analysis

The following is a description of an error analysis procedure derived specifically for this experiment. This equation calculation was incorporated in an effort to provide the investigator an awareness of the error associated with the performance of the measurements. Errors associated with instrumentation can be both mechanical and human. This error, determined as total error ( $C_{ET}$ ), represents the maximum error for an instrument.

$$C_{ET} = \sqrt{u/u + q/q + t/t}$$

where

- $^u$  = error in mass measurement (syringe),
- $^q$  = error in flow rate (rotameter),
- $t$  = error in time.

As delineated above, standard bags were prepared by injecting known volumes of CPFB into Tedlar™ bags. Table 5.4-2 lists the injection volumes and the size of bags used. One and 10- $\mu$ L glass syringes with stainless steel plungers were used in making the injections. The error associated with each is 1% of full scale, or 0.01 and 0.1  $\mu$ L, respectively. The accuracy of the rotameter was determined from the calibration curve to be 0.04 L/min. The air flow into the Tedlar™ bag was timed using a standard laboratory timer (GraLab, Centerville, OH). This timer is accurate to 0.015% for 10 sec, which has been deemed negligible.

TABLE 5.4-2. STANDARD BAG PREPARATION

Concentration (mg/m <sup>3</sup> )	Syringe Volume ( $\mu$ L)	Bag Size (L)
300	9.57	50
125	3.93	50
25	0.80	50
7.5	0.48	100

## RESULTS

Mean concentration values, determined from the linear curves and the associated statistics are presented in Table 5.4-3. The concentration for each day was reported as a time-weighted average. The mean concentrations for the 67 days of exposure were 10.1, 50.4, and 251.4 mg/m<sup>3</sup> with standard deviations of  $\pm 0.7$ , 1.4, and 4.5, respectively. The coefficients of variation (CV) for the chambers from low to high were 6.6, 2.8, and 1.8, respectively.

TABLE 5.4-3. SUMMARY OF CONCENTRATION OF CPFB

Parameter	10 mg/m <sup>3</sup>	50 mg/m <sup>3</sup>	250 mg/m <sup>3</sup>
N	65	65	67
Mean	10.1	50.4	251.4
SD	0.7	1.4	4.5
Max	12.5	54.9	269.8
Min	8.3	47.5	240.6
CV	6.6	2.8	1.8

In addition to chamber concentration determination, other chamber environmental conditions were monitored. Each chamber was equipped with a relative humidity (RH)/temperature sensing unit (HY-Cal Engineering, El Monte, CA). The signal from this instrument was received by the THRU Chamber Data Acquisition System. The RH and temperature were monitored during both exposure

and nonexposure periods. In addition chamber flow rate and chamber pressure also were recorded. All parameters were transferred into a VAX computer 45 min postexposure for data reduction. The mean temperature and RH for each day are reported in Table 5.4-4.

TABLE 5.4-4. RELATIVE HUMIDITY AND TEMPERATURE FOR THE 10-, 50-, AND 250-mg/m<sup>3</sup> EXPOSURE LEVELS

Parameters	10 mg/m <sup>3</sup>		50 mg/m <sup>3</sup>		250 mg/m <sup>3</sup>	
	Temp (°F)	RH (%)	Temp (°F)	RH (%)	Temp (°F)	RH (%)
N	66	66	66	66	66	66
Mean	74.3	67.4	73.7	75.4	73.8	65.8
SD	2.0	7.8	2.1	9.1	2.2	7.2
Maximum	78.6	82.8	78.4	87.7	79.3	74.5
Minimum	69.4	42.8	69.4	46.4	69.1	42.3
CV	2.7	11.6	2.8	12.1	3.0	10.9

The results of the error analysis, Table 5.4-5, indicate that the percent error was greatest for the 25-mg/m<sup>3</sup> bag.

TABLE 5.4-5. RESULTS OF ERROR ANALYSIS FOR THE PREPARATION OF STANDARD BAGS

Target Concentration (mg/m <sup>3</sup> )	Error (mg/m <sup>3</sup> )	Percent Error
300	4.2	1.4
125	3.6	2.9
25	3.2	12.8
7.5	0.19	2.5

#### DISCUSSION

Atmospheres containing CPFB were successfully generated as a homogeneous vapor. The mean concentration values for the 67 days of exposure were within 1% of the target concentrations. The CV for the 10-mg/m<sup>3</sup> level was higher than the other two test levels. This can be partially attributed to the behavior of the THRU chambers and to the sensitivity of the analytical technique. In addition, the variability in chamber concentration also may be partially attributed to error in making the standard bags. Based on the error analysis performed, the percentage of error for the 10-mg/m<sup>3</sup> level chamber was 12.8%. The other chambers were shown to have less than 3.0% error.

Changes in room temperature affected generator output, usually resulting in increased chamber concentration. Compensation for these fluctuations in chamber concentration were made by increasing chamber exhaust flow.

## **5.5 OPTIMIZATION OF CUSTOM LATEX MASK CHARACTERISTICS FOR USE IN PRIMATE CPFB RESPIRATION STUDIES**

**W.D. Crank and A. Vinegar**

### **ABSTRACT**

Latex face masks, which will be used in pharmacokinetic studies of chloropentafluorobenzene (CPFB), introduce a confounding element by absorbing CPFB themselves. Approaches that have been considered for minimizing mask absorption are (1) coat the latex mask with a nonabsorbent material, (2) find a commercially available respirator mask made of nonabsorbent material, and (3) place a nonabsorbent conduit inside the latex mask. Absorption of CPFB by various coatings, commercial masks, and other candidate materials was investigated. A material found to be relatively nonabsorbent was a flexible, bonded, five-layer composite with one layer of aluminum. This composite can be used to make a narrow, tubular conduit for conveying gas within the latex mask. Computer simulations predict such a modified latex mask to have greatly reduced absorbency compared to an unmodified mask.

### **INTRODUCTION**

The chemical CPFB has been chosen as a candidate simulant for CW agents. Such a simulant will be used to test an individual's use of protective gear to prevent exposure to volatile toxicants. During a training exercise the innocuous simulant will be used instead of a true warfare agent; after the exercise the dose received by the individual will be ascertained. CPFB has properties that make it suitable to be used in this way. As a vapor, CPFB is readily absorbed and released during respiration (Vinegar et al., In Press). CPFB is relatively nontoxic (Kinkead et al., 1987), and it is metabolized slowly (Jepson et al., 1985).

Before using CPFB with humans, inhalation experiments with rhesus monkeys will be performed. The primary purpose of these experiments is to verify that physiologically based pharmacokinetic (PB-PK) modeling techniques (Gargas and Andersen, 1988), which describe CPFB kinetics in the rat (Vinegar et al., In Press), can be applied to a nonhuman primate. In addition, these experiments provide an opportunity to develop and test the characteristics of apparatus similar to that which will be used in troop training exercises.

For 15 min, monkeys will breathe air containing 300 ppm CPFB vapor, followed by 15 min of air without CPFB. During the initial 15 min, the amount by which CPFB concentration in expired breath is reduced from 300 ppm reflects the absorption of CPFB by the monkey and by the apparatus; subsequently, the amount by which CPFB concentration is elevated above 0 ppm reflects the release of CPFB by the monkey and by the apparatus. In order that measurements intended to reflect CPFB

uptake and release by the monkey not be confounded by uptake and release by the apparatus, it is necessary that absorption by the apparatus be minimized. Valves, connectors, and tubing that convey respired air to and from the monkey have been made of stainless steel and aluminum, which do not absorb CPFB. However, the monkey will breathe through a face mask that is custom made of latex rubber, and latex readily absorbs CPFB.

To reduce CPFB absorption by face masks, three possibilities have been investigated: (1) coat the inside surface of the latex mask with a material that neither absorbs CPFB nor allows penetration of CPFB to the rubber, (2) find a commercial, off-the-shelf respirator mask (an infant size would be fitted to a rhesus monkey) that is made of a material that does not significantly absorb CPFB, and (3) modify the latex mask by putting a narrow, tubular conduit into it. Respired air would pass through the conduit within the mask and would be exposed to nonabsorbing conduit material, not to latex. A suitable material from which to make the conduit would have to be found. All three approaches require that materials be tested for absorption of CPFB.

#### METHODS

Tests for CPFB absorption were performed on latex rubber samples cut from custom masks, on latex rubber samples that had been coated, on samples cut from commercial respirator masks, and on samples of other materials. Each sample had a total surface area of about 4 cm<sup>2</sup>. For each material, absorption of CPFB was tested in a run of about 25 samples. Each sample was placed in a separate glass vial (31.3 mL). One milliliter of air containing CPFB vapor was injected into each vial to produce a consistent initial CPFB concentration within different vials of a single run. Initial intravial CPFB concentration varied between runs, ranging from 200 to 400 ppm. Vials of a single run were incubated for different intervals ranging up to 2 h. After incubation, a 1-mL sample of headspace gas was withdrawn from a vial and tested for CPFB concentration (HP 5880 gas chromatograph). Headspace concentrations decreased with increasing incubation time as more CPFB was absorbed by the sample. From such data, the values of two parameters were determined in order to concisely describe the absorption properties of the sample material. A rate constant characterized the rate of CPFB absorption by the sample – the greater the rate constant, the greater the absorption rate. A partition coefficient characterized the partition of CPFB between air and sample – the greater the partition coefficient, the greater the amount of CPFB absorbed by the sample.

A simple model representing absorption of CPFB by a sample was used to infer the rate constant and partition coefficient from the data. Assumptions of the model were (1) the rate of CPFB absorption by a sample was proportional to the difference between CPFB concentration in air and the CPFB concentration in the sample divided by its partition coefficient, (2) the rate of CPFB absorption was proportional to sample surface area, and (3) CPFB was distributed uniformly throughout the

volume of the sample. The rate constant and partition coefficient appear explicitly in the mathematical expressions of the model. Changing these parameters in the model changes the results of a simulation. The values of the rate constant and partition coefficient that were taken to characterize a sample were those that resulted in the best fit of a simulation to experimental data. The best fit was obtained by using the optimize feature of SimuSolv modeling and simulation software (Mitchell & Gauthier Associates, Concord, MA), which was run on a VAX 8530 computer (Digital Equipment Corporation, Maynard, MA).

A model for absorption of CPFB by a face mask was constructed. For a given mask material, the rate constant for the mask was scaled up from the rate constant for a sample according to surface area exposed to CPFB. The partition coefficient for the mask was the same as that of the sample.

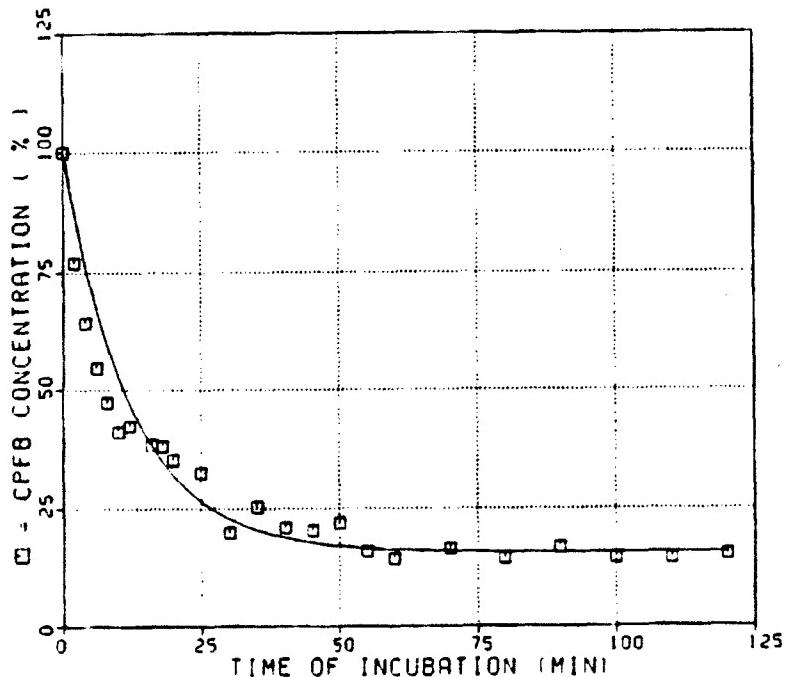
The face mask model was combined with a PB-PK model for the monkey in order to simulate CPFB kinetics in the mask-monkey system. The PB-PK model for the monkey was based on a PB-PK model for the rat. To scale the model from rat to monkey, published data for rhesus monkeys were used for the size of model compartments (Hoffbrand and Forsyth, 1969), the distribution of cardiac output to those compartments (Forsyth et al., 1968), and the ventilation rate (Bourne, 1975). Partition coefficients for CPFB in the various tissues of the monkey were assumed to be the same as those of the rat (Jepson et al., 1985). Simulations of the mask-monkey system, implemented with SimuSolv software on the VAX computer, showed that the magnitude of CPFB concentration changed in inhaled and exhaled gas as it passed through the face mask.

## RESULTS

Absorption of CPFB by latex samples cut from a face mask is shown in Figure 5.5-1. Plotted points are measured CPFB concentrations in headspace gas for different incubation intervals. The curve through the points is from a simulation using a rate constant and a partition coefficient that have been adjusted to optimize the model's fit to the data. In control runs without a sample in the vial, there was no decrease in headspace CPFB concentration. Consequently, absorption by the latex samples entirely accounted for the decrease in CPFB concentration. A latex sample having a 0.4-mL volume absorbed sufficient CPFB to reduce the concentration in 30.9 mL of headspace gas to 20% of its initial value, which indicates that latex has a high affinity for CPFB. The partition coefficient was found to be 376; the rate constant was 0.0021 L/min.

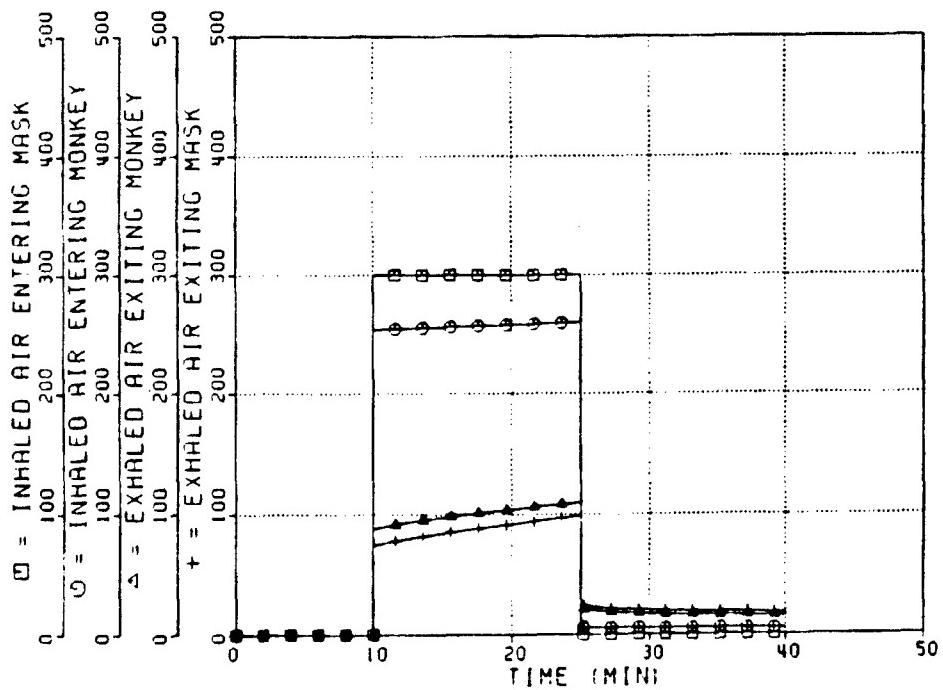
A simulation of a system comprising a monkey breathing through a latex face mask is shown in Figure 5.5-2. During the initial 10 min, air without CPFB was breathed; during the subsequent 15 min, air with 300 ppm CPFB was breathed; during the final 15 min, air without CPFB was again breathed. There are four plots of CPFB concentration against time. The highest curve (during CPFB administration) represents inhaled air entering the mask; the next curve represents air that has

passed through the mask as it enters the monkey; the next curve represents exhaled air as it enters the mask; the lowest curve represents exhaled air leaving the mask. Each successive decrement in CPFB concentration was due to absorption by either the mask or the monkey. Elevation of CPFB concentrations above zero during the final 15 min was due to off-gassing by either the monkey or the mask. It is evident that CPFB levels were altered significantly by the latex mask.



**Figure 5.5-1. Absorption of CPFB by Latex Samples.** Plotted points are measured CPFB concentrations (percent of initial concentration) in headspace gas for incubations up to 2 h. The curve has been fitted to the points by optimizing rate constant and partition coefficient.

The simulation illustrated in Figure 5.5-2 has motivated efforts to find a way to minimize CPFB absorption by the face mask. Several coating materials that might be applied to the inside surface of the mask have been tested. Metallic spray paints purchased at a local hardware store were rejected because they did not adhere well to the latex. A local plating shop applied a thin layer of Teflon® to one set of latex samples and a metallic lacquer to another set of latex samples. There was no adhesion problem with either of these two coating materials, and both sets of coated samples were tested for CPFB absorption. Table 5.5-1 presents the values obtained for partition coefficient and absorption rate constant. Neither Teflon® nor metallic lacquer blocks CPFB absorption significantly.



**Figure 5.5-2. Simulation of a Monkey Breathing through a Latex Mask.** Breathing air with 300 ppm CPFB for 15 min is preceded and followed by breathing fresh air. The time course of CPFB concentration is shown for (a) inhaled air entering the mask, (b) inhaled air leaving the mask while entering the monkey, (c) exhaled air leaving the monkey while entering the mask, and (d) exhaled air leaving the mask.

**TABLE 5.5-1. CPFB ABSORPTION PROPERTIES OF SAMPLES OF POTENTIAL MASK MATERIALS**

Material	Partition Coefficient	Rate Constant (L/min)
<b>Custom latex mask</b>		
Latex	376	0.00213
Latex & Teflon®	297	0.00181
Latex & metallic lacquer	186	0.00206
<b>Respirator masks</b>		
Butyl rubber	147	0.00161
Silicone rubber	326	0.02145
Natural rubber #1	196	0.00184
Natural rubber #2	148	0.00132
<b>Other materials</b>		
Neoprene	97	0.00162
Buna-n	87	0.00062
Nitrile	63	0.00014
Five-layer composite	16	0.00039

The possibility of fitting a commercially available respirator mask (infant size) to the rhesus monkeys has been considered. Samples cut from masks made of silicone rubber, butyl rubber, and natural (latex) rubber have been tested for CPFB absorption. Partition coefficients and rate constants for these samples, which are given in Table 5.5-1, indicate sufficient absorption to preclude use of these masks.

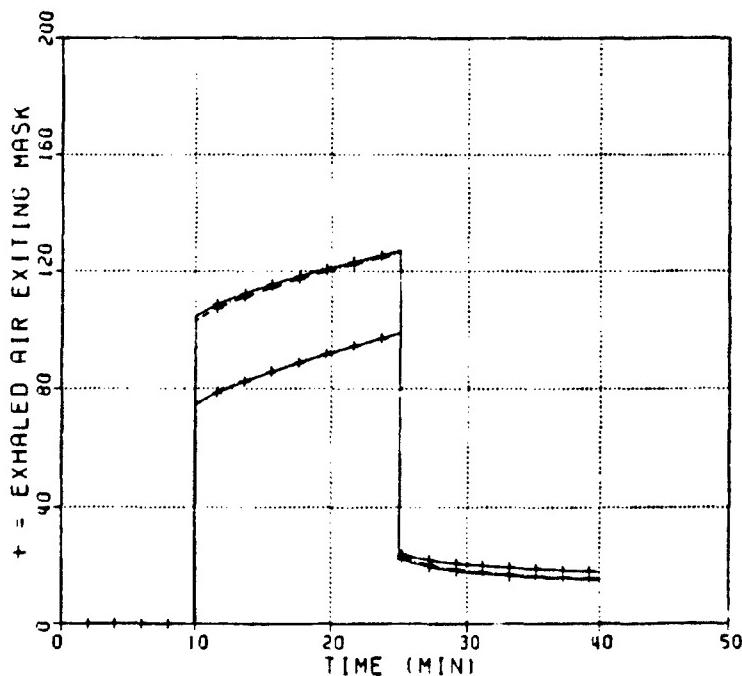
Samples of other materials also were tested for CPFB absorption (Table 5.5-1). The least absorbent is a flexible, 5.5-mil thick, five-layer bonded composite (available from Calibrated Instruments, Inc., Ardsley, NY) made of polyethylene, polyamide, aluminum, polyvinylchloride, and polyester. This nonabsorbency will be exploited by placing an internal conduit, made of an aluminum tube and the composite, within a latex mask. Through the tubular conduit, resired gas will pass between a nonrebreathing valve at one side of the mask and the monkey's mouth and nostrils at the other side of the mask. Because the conduit is made of nonabsorbent materials, and because the surface area of the conduit is less than that of the latex mask, absorption of CPFB by the latex mask is expected to be reduced greatly.

Simulations of the system comprising mask and monkey predict improved performance due to the modification. The paradigm of 15 min of exposure to 300 ppm of CPFB followed by 15 min of no CPFB has been used. For three different masks, the three curves of Figure 5.5-3 illustrate computed CPFB concentration of exhaled air leaving the mask. The highest curve (during the 15 min of exposure) is for an ideal mask that absorbs no CPFB; the lowest curve is for an unmodified latex mask; the middle curve is for a modified mask with a conduit. After exposure is terminated, the order of the curves is reversed. Although the unmodified latex mask absorbs considerable CPFB, the modified mask performs almost as well as an ideal, nonabsorbing mask.

#### DISCUSSION

Assuming uniform CPFB concentration throughout a sample implies a monoexponential headspace curve. A biexponential curve, which would fit the data of Figure 5.5-1 slightly better, implies a two-compartment representation of the sample. The slight improvement to be obtained with a two-compartment representation did not justify additional effort to investigate intercompartmental diffusion of CPFB in latex rubber.

The simulations predict that a modified mask will absorb much less CPFB than an unmodified latex mask. To verify this prediction, it is planned to do experiments in which a mask and surrogate monkey (nonabsorbing bellows) are placed in the setup and CPFB concentrations in inhaled and exhaled air are measured. If the modified mask absorbs negligibly, as expected, then experiments on monkeys can proceed without a confounding effect due to the mask.



**Figure 5.5-3. Simulations of the Monkey-Mask System Showing CPFB Concentration in Exhaled Air Leaving the Mask.** Results for three masks are shown. During exposure, the top curve represents an ideal nonabsorbent mask; the bottom curve represents an unmodified latex mask; the middle curve represents a latex mask that has been modified by placing a nonabsorbent conduit into it. After termination of exposure, the order of the curves is reversed.

Troop training exercises done with a simulant to test effective use of protective gear will require individuals to breathe into an apparatus that measures exhaled simulant levels. However, absorption by a face mask on that apparatus will alter simulant levels between the subject and the measurement site. To circumvent that problem, face masks for humans could be modified in the same manner as face masks for monkeys.

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## 5.6 GENOTOXICITY POTENTIAL OF CHLOROPENTAFLUOROBENZENE AND DICHLOROTETRAFLUOROBENZENE

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### ABSTRACT

Two fully substituted halogenated benzenes, chloropentafluorobenzene (CPFB) and dichlorotetrafluorobenzene (DCFB) were evaluated in *in vitro* bioassays to assess their potential genotoxic activity. The assays conducted were the Ames *Salmonella*/microsomal mutagenicity assay, the Chinese hamster ovary (CHO) cell gene mutation assay using the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus, the CHO/sister chromatid exchange (SCE) and chromosome aberration assay, and the BALB/c-3T3 cell transformation assay. CPFB did not demonstrate genotoxic potential in any assays with the exception of the SCE assay in the presence of metabolizing enzymes. DCFB proved negative in all of the assays conducted.

### INTRODUCTION

Chloropentafluorobenzene and DCFB are fully substituted halogenated benzenes. These compounds are being considered as test agents to assess the effectiveness of chemical defense procedures and equipment. It is anticipated that when used in training exercises the breath of trainees will be analyzed for the training agent and estimates will be made of what their dose of a chemical agent would have been. To fully evaluate the safety of these proposed training agents *in vitro* and *in vivo/in vitro* tests were conducted to determine their genotoxic potential.

### MATERIALS AND METHODS

The *Salmonella* reverse mutation assay (Ames et al., 1975), using a preincubation method (Yahagi et al., 1975) was employed to examine mutagenic activity. This assay evaluated the test articles and/or their metabolites for ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes. The tester strains used in this study were TA98, TA100, TA1535, TA1537, and TA1538. Following toxicity testing, seven concentrations of CPFB were tested, from 10.0 to 10,000 µg/plate in the presence of S9 and from 5.00 to 5,000 µg/plate in the absence of S9. The concentrations of DCFB tested ranged from 1.0 to 2,500 µg/plate in the absence of S9 fraction and 5.0 to 5,000 µg/plate in the presence of S9 fraction. By convention the doses have been expressed as micrograms of test article per plate to reflect the fact

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that exposure of the test system to the test article did not cease at the end of the 20-min preincubation period.

To evaluate the ability of these chemicals to induce forward mutations in mammalian cells, the HGPRT locus in CHO cells, was used in an *in vitro* assay both with and without metabolic activation. This assay was conducted using glass culture vessels and Pluronic® F-68, a biologically compatible surfactant. The results from the preliminary range-finding cytotoxicity assays were used to select concentrations for the mutation assays. Treatment conditions chosen for both the nonactivation and S9 metabolic activation portions of the CPFB mutation assay ranged from 0.01 to 1.0 mg CPFB/mL. Treatment conditions selected for the nonactivation portion of the DCFB mutation assay ranged from 0.005 to 1.0 mg/mL and for the activation portion the treatment conditions ranged from 0.01 to 1.0 mg/mL. The assay procedures were based on those reported by Hsie et al. (1975), and reviewed by Hsie et al. (1981), with modifications suggested by Myhr and DiPaolo (1978).

In the SCE assay, conducted in glass vessels, CHO cell cultures were exposed to the test article, which was emulsified using Pluronic® F-68 for approximately two cell cycles. The cultures were analyzed to determine cellular toxicity and effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to allow for the progression to second generation cells. The doses used in the assay ranged from 0.167 to 5010 µg/mL in a half-log series for both CPFB and DCFB.

Cell cycle kinetics from the SCE assay were used to (1) determine the dose range to be used in the chromosomal aberrations assay and (2) determine the optimal time of harvest of the dosed cells so that primarily metaphase cells, which were in the first metaphase since exposure to the test article, would be analyzed for chromosomal aberrations. For both CPFB and DCFB, 10- and 20-h harvests were conducted for the nonactivation assay and 10-h harvests were conducted for the activation assay. Chromosomal aberrations were analyzed from the four highest doses from which results could be obtained and from only one of the positive control doses.

These materials were assayed for their ability to induce morphological cell transformation in BALB/c-3T3 cell cultures in the absence and presence of a rat liver S9 metabolic activation system. Seven concentrations of CPFB (50 to 600 µg/mL) and six concentrations of DCFB (25 to 250 µg/mL) were examined as emulsions in Eagle's minimum essential medium containing 1% w/v Pluronic® F-68. Toxicity was determined from the clonal survival of ouabain-resistant cells in the presence of a wild-type monolayer.

The BALB/c-3T3 mouse cell transformation assay procedures were adapted from those reported by Kakunaga (1973). Glass culture bottles having a monolayer growth area of approximately 60 cm<sup>2</sup> were used. Prior to the transformation assays, cytotoxicity tests were conducted to establish the appropriate concentrations of test material. The transformation assays for CPFB, both with and without metabolic activation, were conducted using concentrations of 50 to 600 µg/mL. The

transformation assays for DCFB without metabolic activation were conducted at concentrations of 25 to 250 µg/mL and assays with metabolic activation of DCFB were conducted at concentrations of 50 to 250 µg/mL.

## RESULTS

### *Ames Salmonella Reverse Mutation Assay*

The *Salmonella* reverse mutation studies conducted on CPFB and DCFB did not result in increases in the mean numbers of revertants per plate with any of the tester strains, either in the presence or absence of S9.

### *HGPRT Mammalian Forward Mutation Assay*

Under nonactivation test conditions, the CHO-K1-BH<sub>4</sub> cultures treated with CPFB showed a dose-related decrease in both relative survival and relative population growth. The cultures exposed to 0.4 and 1.0 mg CPFB/mL were completely killed and were terminated. An intermediate dose, 0.5 mg/mL, had less than 10% relative clonal survival. Seven CPFB doses (0.01 to 0.5 mg/mL) were available for analysis and the mutant frequency of the treated cultures varied within the acceptable range of vehicle control mutant frequencies (0 to  $15 \times 10^{-6}$ ). There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was both significantly elevated over the average background mutant frequency of the concurrent vehicle controls and higher than the acceptable range of background mutant frequencies.

Two independent mutation assays were performed with CPFB using activation conditions. In each trial dose-related toxicity was observed. In the first trial, 10 concentrations were used that ranged from 0.01 to 1.0 mg CPFB/mL. The mutant frequencies of treated cultures were within the acceptable range (between 0 and  $15 \times 10^{-6}$ ) for background mutant frequency variation with the exception of the culture treated with 0.5 mg CPFB/mL. That culture had a mutant frequency of  $17.4 \times 10^{-6}$  that also was elevated statistically over the mutant frequencies of the concurrent vehicle control cultures. The mutant frequencies of the other cultures varied randomly with dose and no other culture had a significantly elevated mutant frequency. Assay evaluation criteria required that a second mutation assay be performed to properly evaluate the elevated mutant frequency at the highest acceptable concentration. The second CPFB metabolic activation mutation assay used a modified dose range to focus on the toxic range where significance occurred in the first trial. Six concentrations were used that ranged from 0.1 to 0.6 mg/mL. The six concentrations analyzed had relative survivals that ranged from 103.2% at 0.1 mg/mL to 12.9% for the culture at 0.5 mg/mL. For unknown reasons the culture treated with the highest concentration, 0.6 mg/mL, was less toxic than the next lower concentration and had 67.5% relative clonal survival. With one exception the mutant frequencies of all the treated cultures varied randomly with dose and were within the range for acceptable background mutant frequencies. The mutant frequency of one of the six cultures in the

second trial was elevated significantly over the mutant frequencies of the concurrent vehicle control cultures, but was within the acceptable background frequency range.

Under nonactivation test conditions, the cultures treated with DCFB showed a dose-related decrease in both relative survival and relative population growth. The highest concentration, 1.0 mg/mL, was completely toxic and the culture was terminated. Of the remaining nine concentrations, the lowest (0.005 mg/mL) was not plated because there was a sufficient number of surviving nontoxic doses. Eight concentrations were available for analysis and the mutant frequency of the treated cultures varied within the acceptable range of the vehicle control mutant frequency (0 to  $15 \times 10^6$ ). There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was both significantly elevated over the average background mutant frequency and the frequency observed in the concurrent vehicle controls.

The cultures treated with DCFB under S9 metabolic activation test conditions showed a dose-related decrease in both relative survival and relative population growth. Excessive toxicity was observed at the two highest concentrations, 0.5 and 1.0 mg/mL, and these were not plated for mutant selection. Of the remaining eight concentrations, the next to the lowest (0.025 mg/mL) was not plated because there was a sufficient number of surviving nontoxic treatment concentrations. Seven doses were available for analysis and the mutation frequency varied within the acceptable range of vehicle control mutant frequency. There was no correlation of mutant frequency with dose and no treated culture had a mutant frequency that was significantly elevated over the average background mutant frequency of the concurrent vehicle control.

#### *Sister Chromatid Exchange Assays*

In the CPFB SCE assay without metabolic activation dead cell monolayers and no visible mitotic cells were observed at concentrations of 501 µg/mL and greater. No significant toxicity was observed at the subsequent lower concentration of 167 µg/mL. Therefore results were evaluated at 5.01, 16.7, 50.1, and 167 µg/mL. Analysis of cell cycle kinetics revealed a slight cell cycle delay at 167 µg/mL. No significant increase in SCE was observed at the concentrations analyzed, except at the 16.7-µg/mL concentration, which was increased significantly over the solvent control culture. However, the change at 16.7 µg/mL was not significantly greater than the negative control or the historical solvent control results (~8.0 SCE/cell).

In the presence of metabolic enzymes complete cytotoxicity was observed again at CPFB concentrations of 501 µg/mL and greater as in the nonactivation assay. No toxicity was discernible at 167 µg/mL. Significant increases in the number of SCE per cell were observed at 16.7, 50.1, and 167 µg/mL (Table 5.6-1). To verify the positive response, this trial was repeated testing concentrations of 50.1, 100, 150, 200, 250, and 501 µg CPFB/plate. Very little toxicity was evident at concentrations of 150 µg/mL and less. Results were analyzed at 100, 150, 200, and 250 µg/mL although cytotoxicity was

evident at the highest two concentrations. A significant dose-related increase in SCE was observed at all the concentrations tested (Table 5.6-1).

**TABLE 5.6-1. EFFECT OF CHLOROPENTAFLUOROBENZENE ON THE FREQUENCY OF SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS IN THE PRESENCE OF METABOLIC ENZYMES**

Treatment	Dose ( $\mu\text{g/mL}$ )	Total Cells Scored	# of Chromo- somes	# of SCE	SCE/ Chromo- some	SCE/Cell Mean $\pm$ SEM	% SCE Increase Over Solvent	Confluence % Solvent Control
<b>Trial 1</b>								
Controls								
Negatives (McCoy's 5a)		50	1029	393	0.38	7.86 $\pm$ 0.43		
Solvent (10% Pluronic <sup>®</sup> )	(100)	50	1042	371	0.36	7.42 $\pm$ 0.42	100	
Positive (CP)	1.50	20	411	606	1.47	30.30 $\pm$ 1.10*	314	100
Test Article (CPFB)	5.01	50	1037	437	0.42	8.74 $\pm$ 0.51	18	100
	16.7	50	1045	449	0.43	8.98 $\pm$ 0.57*	21	100
	50.1	50	1045	461	0.44	9.22 $\pm$ 0.61*	24	100
	167	50	1045	553	0.53	11.06 $\pm$ 0.53*	50	100
	501**						13	
<b>Trial 2</b>								
Controls								
Negatives (McCoy's 5a)		50	1046	415	0.40	8.30 $\pm$ 0.44		
Solvent (10% Pluronic <sup>®</sup> )	(10)	50	1045	372	0.36	7.44 $\pm$ 0.40	100	
Positive (CP)	1.50	20	418	1054	2.52	52.70 $\pm$ 2.98*	608	88
Test Article (CPFB)	100	50	1050	465	0.44	9.30 $\pm$ 0.41*	24	88
	150	50	1049	513	0.49	10.26 $\pm$ 0.54	37	75
	200	50	1046	609	0.58	12.18 $\pm$ 0.58*	64	50
	250	50	1045	678	0.65	13.56 $\pm$ 0.70*	82	13
	501**						0	

\*Significantly greater than the solvent control at  $p < 0.01$  using Student's t-test corrected for multiple comparisons.

\*\*Toxic dose

CP = Cyclophosphamide

CPFB = Chloropentafluorobenzene

In the DCFB SCE assay without metabolic activation, complete cellular toxicity was observed at 167, 501, 1670, and 5010  $\mu\text{g/mL}$ . Results were evaluated at 1.67, 5.01, 16.7, and 50.1  $\mu\text{g}$  DCFB/plate. A weakly positive response was observed at 50.1  $\mu\text{g}/\text{mL}$ . The assay was repeated testing concentrations of 10.0, 25.0, 50.1, 75.1, and 100  $\mu\text{g}$  DCFB/plate. A dead cell monolayer was observed at 100  $\mu\text{g}/\text{mL}$ . An unhealthy cell monolayer, a severe reduction in visible mitotic cells, and ~60% reduction in the cell monolayer confluence were observed at 75.1  $\mu\text{g}/\text{mL}$ . Results were evaluated at 10.0, 25.0, 50.1, and 75.1  $\mu\text{g}$  DCFB/plate and no significant increase in SCE was observed.

In the presence of metabolic enzymes complete cytotoxicity was observed at DCFB concentrations of 167 µg/mL and greater. Results were evaluated at 1.67, 5.01, 16.7, and 50.1 µg DCFB/plate and a weakly positive response was again observed at 50.1 µg/mL. This response was investigated testing concentrations of 10.0, 25.0, 50.1, 75.1, and 100 µg DCFB/plate. Evaluation revealed a weakly positive increase in SCE only at 100 µg DCFB/plate.

#### ***Chromosomal Aberrations Assays***

Cell cycle kinetics from the nonactivation CPFB SCE assay indicated a slight cell cycle delay at 167 µg CPFB/mL. Based on this observation, two harvest times were selected for the aberration assay. A 10-h harvest was selected for testing concentrations of 20.0, 50.1, 100, 150, and 200 µg/mL and a 20-h harvest was selected for testing concentrations of 200, 250, 300, 400, and 501 µg/mL. No significant increase in chromosomally aberrant cells was observed at the concentrations analyzed with the 10- or 20-h harvest.

In the CPFB SCE assay with metabolic activation, no cell cycle delay was evident at the doses analyzed and a 10-h harvest was selected for testing concentrations of 50.1, 100, 150, 200, 250, 300, 400, and 501 µg CPFB/plate. Complete cellular toxicity was observed at 300, 400, and 501 µg/mL. Therefore, results were evaluated at 100, 150, 200, and 250 µg/mL and no significant increase in chromosomally aberrant cells was observed.

Based on the cell cycle kinetic data from the DCFB nonactivation SCE assay, 10- and 20-h harvest times were selected for the aberrations assay. DCFB concentrations of 6.26, 12.5, 18.8, and 25.1 µg/mL were tested with a 10-h harvest and concentrations of 25.0, 50.1, 75.1, and 100 µg/mL were tested with a 20-h harvest. No significant increase in chromosomally aberrant cells was observed at the concentrations analyzed.

In the DCFB SCE assay with metabolic enzymes, no cell cycle delay was observed at the concentrations assessed. A 10-h aberrations assay was selected testing concentrations of 7.51, 11.3, 15.0, 37.5, 75.1, 113, and 150 µg DCFB/plate. Results were evaluated at 15.0, 37.5, 75.1, 113, and from a single surviving culture at 150 µg/mL and no significant increase in chromosomally aberrant cells was observed.

#### ***BALB/c-3T3 Transformation Assay***

At concentrations of 625 µg/mL and greater, CPFB was toxic or lethal to BALB/c-3T3 cells. Little or no toxicity was evident at 156 µg/mL. These results were essentially unchanged by the addition of the rat liver S9 metabolic activation system. Thus, a dose range of 100 to 600 µg/mL covered the entire survival range appropriate for the transformation assay with or without S9. Six doses were chosen at 100 µg/mL increments over this range, and a seventh dose (50 µg/mL) also was included to help define the variation in response for nontoxic treatments.

For treatments with 50 to 500 µg CPFB/mL (approximately 100% survival to 2% survival) in the absence of metabolizing enzymes, the frequency of transformed foci remained equivalent to the solvent control. An apparent increase in transformed foci was observed in one culture but the total number of foci still remained below that observed in the negative control cultures.

In BALB/c-3T3 cultures in the presence of metabolizing enzymes the frequency of transformed foci in the cultures exposed to CPFB remained strictly comparable to the solvent or medium negative controls. An apparent increase for the nontoxic treatment with 200 µg/mL was due to one culture. No increases were observed for the toxic range of treatments (400, 500, and 600 µg/mL).

DCFB was found to be essentially lethal at 300 µg/mL and to have little or no toxicity at 50 µg/mL both with and without the addition of metabolizing enzymes. Therefore, five doses were chosen at 50 µg/mL increments from 50 to 250 µg/mL, and a sixth dose (25 µg/mL) was included to help define the variation in response for nontoxic treatments.

In the absence of metabolizing enzymes, DCFB treatment of BALB/c-3T3 cells at concentrations of 25 to 250 µg/mL (92 to 9% survival) did not result in an increased frequency of transformed foci relative to solvent and medium controls. In the presence of S9 fraction the frequency of transformed foci in DCFB-treated cultures remained comparable to those of the solvent and culture medium negative controls. An apparent increase observed at the relatively nontoxic treatment of 100 µg DCFB/plate was due to one culture that contained five foci. However, there was no increase observed at the higher concentrations of 150, 200, and 250 µg/mL which spanned the entire survival range.

#### DISCUSSION

CPFB and its potential metabolites were found not to have genotoxic potential under the conditions of the *Salmonella* reverse mutation, HGPRT mammalian cell mutation, chromosomal aberration, and the BALB/c-3T3 transformation assays. The HGPRT mutation frequency of CHO cultures treated with CPFB in the presence of S9 were within the acceptable background range with one exception and this result prompted a second trial. The significant mutant frequency seen at 0.5 mg CPFB/plate in the first trial was not confirmed at the same concentration in the second trial. Although another culture exhibited a mutation frequency significantly increased over the concurrent control it was within the acceptable background range. The significant differences observed in mutant frequency were apparently the result of normal assay variation. Therefore, CPFB was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells under the S9 metabolic activation conditions used in the study.

CPFB was considered negative for inducing sister chromatid exchanges in CHO cells under the nonactivation conditions of this assay. However, CPFB did increase the mean number of SCE in CHO cells when metabolizing enzymes were present in the culture media. The results from a single culture in the initial trial prompted a second trial that resulted in a positive dose response from 100 to 250 µg

CPFB/plate. CPFB had been reported to cause increased numbers of SCEs in CHO cells both with and without metabolic activation (Steele, 1987). In a study by Tu et al. (1986), CPFB was indicated as positive in the SCE assay only in the absence of metabolic enzymes. In studies where CPFB has induced increased SCE the minimum concentration has been from 100 to 300 µg/mL and it remains unclear as to what type of exposure scenario would result in such a tissue concentration. In a 21-day, 6 h/day inhalation study that included B6C3F<sub>1</sub> mice exposed to 0.25, 0.80, or 2.50 mg CPFB/L, no change was observed in the incidence of SCE (Kinkead et al., 1989).

DCFB and its potential metabolites were found not to have genotoxic potential under the conditions of the *Salmonella* reverse mutation, HGPRT mammalian cell mutation, SCE, chromosomal aberration, and the BALB/c-3T3 transformation assays. The most notable response observed in the tests conducted on DCFB was variability in the cell cycle kinetics in the CHO SCE and chromosomal aberration assays. The DCFB emulsion may not have interacted uniformly with cell monolayers in this assay and variable toxicity may have resulted. However, the material was present in the cultures for up to 20 h with cells in S and G phases and the assays were considered valid.

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## SECTION 6

### NAVY TOXICOLOGY INVESTIGATIONS

#### 6.1 THE ACUTE DELAYED NEUROTOXICITY EVALUATION OF TWO JET ENGINE OIL FORMULATIONS

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##### **ABSTRACT**

This study was designed to determine the potential of two jet engine oils to produce acute, delayed neurotoxicity. The hydrocarbon-based ester oil formulation contained 3% tricresyl phosphate (TCP) isomers including triorthocresyl phosphate (TOCP) in one of the formulations. Hens were dosed orally over a five-day period and then were observed for a total period of 30 days. All TOCP-positive control hens demonstrated signs of acute, delayed neurotoxicity. Hens from both jet engine oil groups remained asymptomatic throughout the observation period.

##### **INTRODUCTION**

The Navy is interested in evaluating the acute, delayed neurotoxicity of two jet engine oil formulations developed by the Mobil Oil Corporation. These samples potentially could result in axonal degeneration characteristic of organophosphate-induced delayed neuropathy (OPIDN) due to a TCP additive. The major component of each oil is a mixture of hydrocarbon-based esters. In the first formulation (NMRI/TD No. 9082-1) the additive is 3% TCP isomers. In the second formulation (NMRI/TD No. 8323-1), the additive is 3% of the ortho derivative of tricresyl phosphate (TOCP), a known neurotoxin. The Navy Medical Research Institute/Toxicology Detachment (NMRI/TD) requested that an acute delayed neurotoxicity study be performed on each of the samples to determine the potential health hazard involved with using these lubricants.

Many organophosphorus compounds have been found to cause delayed neurotoxic effects in man (Norton, 1980). A single exposure to a neurotoxic organophosphorus compound has been reported to be capable of producing axonal damage after a delay of eight to 10 days. Low-level nerve injury may occur in humans after chronic exposure to these compounds. Similar neurotoxic effects have been demonstrated in adult chickens and cats after exposure to TOCP (Beresford and Glees, 1963). Organophosphorus compounds that cause axonal pathology interact with the enzyme neurotoxic esterase (NTE) in the initial step of the delayed neurotoxicity. This interaction occurs within hours of dosing and can be measured in the brain by the NTE assay. NTE inhibition in brain correlates with that in the spinal cord and nerve (Davis and Richardson, 1980).

This study was designed to determine if delayed neurotoxic effects result from exposure of adult chickens to the two jet engine oils of interest (NMRI/TD Nos. 9082-1 and 8323-1). Both engine oil samples, as well as a vehicle control and a TOCP-positive control, were tested concurrently. Final determination of an injury effect was based on a comparison of the test chickens with the TOCP-control chickens. An NTE assay was performed on a portion of the test animals for comparison with the nonbiochemical assay.

#### TEST MATERIALS

The two engine oil samples supplied by NMRI/TD are listed below.

Jet engine oils:

**No. 9082-1**

Additive: 3% TCP

Density: 1.003 g/mL at 60 °F

Initial Boiling Point: >600 °F

Vapor Pressure: <0.1 mm Hg at 37.8 °C

**No. 8323-1**

Additive: 3% TOCP

Density: 1.003 g/mL at 60 °F

Initial Boiling Point: >600 °F

Vapor Pressure: <0.1 mm Hg at 37.8 °C

Positive and negative control materials:

Triorthocresyl phosphate, practical grade,  
Lot # C98 obtained from Eastman Kodak Co.,  
CAS # 78-30-8.

Corn oil, commercial grade, purchased locally. The corn oil was tested for the presence of peroxides prior to use.

An infrared (IR) spectrum of each sample was obtained prior to the start of the study. The THRU/NSI-ES Chemistry Section retained an archive sample of each test material.

#### ANIMALS

Delayed neurotoxicity potential was evaluated using leghorn hens (*Gallus domestica*, Carey Nick 320 hybrid, Carey Farms, Inc., Marion, OH), eight to 14 months of age, weighing between 1.1 and 2.1 kg. The debeaked hens were identified by leg bands and were group housed in 3 ft x 6 ft pens to allow free movement. Food (MannaPro, Eggmaker 15 Crumbles) and water were provided ad libitum. Hens were maintained on a 15-h light/dark cycle starting at 0300 h.

Verbal communication with the supplier (Carey Farms, Inc.) provided additional flock history and husbandry practices. Table 6.1-1 lists the vaccinations administered to the flock. No pesticides were applied to hens used in this study, nor were disinfectants used while the birds were in the

poultry houses. When poultry houses became vacant, they were cleaned and disinfected with formaldehyde. The supplier indicated that the flock had not experienced any disease problems.

TABLE 6.1-1. FLOCK VACCINATION HISTORY<sup>a</sup>

Vaccination	Age of hen
Marek's disease	1 day
Infectious bronchitis	2 weeks
Infectious bursal disease	2 weeks
Newcastle disease	2 weeks
Infectious bronchitis (booster)	10-12 weeks
Newcastle disease (booster)	10-12 weeks
Fowlpox	20-24 weeks

<sup>a</sup>Provided verbally by Carey Farms, Inc., Marion, OH.

#### ACUTE, DELAYED NEUROTOXICITY

The design of this study followed the requirements of military specification MIL-H-19457B. All hens were weighed prior to the start of the study and weekly thereafter. Test substances were administered to unfasted adult hens by oral intubation employing a 3-cc syringe fitted with a 15-cm infant feeding catheter. Doses were administered on five consecutive days beginning on Monday. The engine oils and TOCP were diluted in corn oil to obtain the appropriate dose. Each hen was weighed prior to the initial dose and a 1.0-mL/kg body weight dose was administered by gavage. The dosing regimen was as follows.

- |                        |  |
|------------------------|--|
| Engine oils            | Groups of four hens each were treated with 420, 360, 300, or 240 mg/kg/day for five days.                    |
| TOCP-positive controls | Groups of four hens each were treated with 90, 75, or 60 mg/kg/day for five days.                            |
| Corn oil               | Twelve hens were given the maximum total volume of fluid equal to that given test animals (i.e., 1.0 mL/kg). |

Observations and scoring began seven days after the first dose and continued three times a week (Monday, Wednesday, and Friday) until 30 days after the initial dose. The following scoring system was used.

Symptom-free	0 points
Doubtful or minor symptoms	2 points
Positive paralytic symptoms	8 points
Advanced paralytic symptoms	12 points
Death	16 points

During observation and scoring, the chickens were removed from their enclosures and placed on a rubber mat to provide sure footing. Symptoms observed in test hens during the observation period were compared with those seen in the TOCP-treated hens.

Reported scores represented an average of the scores of three observers. The mean symptom scores noted on Day 21 after the initial dose were used to calculate a TOCP equivalent as required by the military specification. This calculation is as follows.

$$TOCP \text{ Equivalent} (\%) = \frac{\text{mg/kg TOCP}}{\text{mg/kg Test Material}} \times \frac{\text{Total Score for Test Material} \times 100}{\text{Total Score for TOCP}}$$

Hens that died during the 30-day study were examined for gross pathology at death. All surviving chickens were sacrificed upon completion of the observation period. The entire brain, spinal cord, and both sciatic nerves (with attached gastrocnemius muscles), were collected for histopathologic examination. Histologic sections were prepared of the medulla, cerebellum, optic lobes, and frontal cortex of the brain; cervical, thoracic, and lumbosacral segments of the spinal cord; the proximal, middle, and distal thirds of one sciatic nerve; the entire gastrocnemius; and any observed gross lesions. Duplicate sciatic nerve and spinal cord sections from at least three hens per treatment group were stained with Bodian's stain to demonstrate cytoplasm in neuron cell bodies and processes, and Luxol Fast Blue to demonstrate myelin.

#### NTE Assay

Additional hens (four per group), as well as an additional treatment level (1000 mg/kg), were used in the NTE assays. Doses were administered in the same manner as that described for the acute, delayed neurotoxicity hens. Twenty-four hours following the fifth treatment all hens were euthanized and the brain of each was removed for the NTE assay. The dosing was as follows.

Jet engine oil samples	Groups of four hens each were treated with 1000, 420, 360, 300, or 240 mg/kg/day for five days.
TOCP-positive controls	Groups of four hens each were treated with 90, 75, or 60 mg/kg/day for five days.
Corn oil	Four hens were given the maximum total volume of fluid equal to that given to test animals (i.e., 1.0 mL/kg).

#### Statistical Analysis

Body weight plus or minus the standard error of the mean (SEM) was calculated using a two-factorial analysis of variance (ANOVA). Body weights were compared using the Ryan-Einot-Gabriel-Welsch Multiple F-test (SAS Institute, Inc., 1985). Fischer's Exact test and the Yates' Corrected Chi-

Square test were used to compare histopathologic lesions (Zar, 1974). The severity of lesions was compared using ANOVA and the Scheffe Multiple Comparison test (Zar, 1974).

#### EXPERIMENTAL RESULTS

The NTE assay was performed by the Mobil Oil Corporation laboratories under an agreement with NMRI/TD. Results of that assay are unavailable and will not be included in this report.

Mean body weights of the hens during the course of the study are listed in Table 6.1-2. All test groups, including the corn oil controls, maintained body weight ( $\pm 5\%$ ) throughout the study. Only the TOCP-treated hen groups showed a decrease in mean body weights throughout the study. Mean body weights of the two higher TOCP-treatment groups were different statistically at three weeks ( $p < 0.05$ ), whereas all were different ( $p < 0.05$ ) at four weeks.

TABLE 6.1-2. EFFECTS OF ORAL INTUBATION OF JET OILS ON CHICKEN BODY WEIGHTS

Treatment Group	Body Weights (kg)			
	Day 0	Day 14	Day 21	Day 28
Corn oil <sup>b</sup>	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.5 $\pm$ 0.1
TOCP (mg/kg)				
90	1.6 $\pm$ 0.1	1.5 $\pm$ 0.2 <sup>c</sup>	1.3 $\pm$ 0.1 <sup>c,d</sup>	1.2 $\pm$ 0.1 <sup>c,d</sup>
75	1.6 $\pm$ 0.1	1.6 $\pm$ 0.2	1.4 $\pm$ 0.1 <sup>d</sup>	1.2 $\pm$ 0.2 <sup>d</sup>
60	1.8 $\pm$ 0.2	1.7 $\pm$ <0.1	1.4 $\pm$ 0.1	1.3 $\pm$ 0.2 <sup>d</sup>
9082 (mg/kg)				
420	1.7 $\pm$ 0.1	1.7 $\pm$ 0.2 <sup>c</sup>	1.7 $\pm$ 0.2	1.7 $\pm$ 0.2
360	1.6 $\pm$ 0.1	1.7 $\pm$ 0.2	1.6 $\pm$ 0.2	1.6 $\pm$ 0.2
300	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1
240	1.6 $\pm$ 0.1	1.7 $\pm$ 0.1	1.7 $\pm$ 0.2	1.7 $\pm$ 0.2
8323 (mg/kg)				
420	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.5 $\pm$ 0.1	1.5 $\pm$ 0.2
360	1.6 $\pm$ 0.1	1.7 $\pm$ 0.1	1.7 $\pm$ 0.1	1.6 $\pm$ 0.1
300	1.6 $\pm$ 0.1	1.8 $\pm$ <0.1	1.7 $\pm$ 0.1 <sup>c</sup>	1.7 $\pm$ 0.1
240	1.6 $\pm$ 0.1	1.6 $\pm$ 0.2	1.5 $\pm$ 0.2	1.4 $\pm$ 0.2

<sup>a</sup> Mean  $\pm$  SEM, N = 4.

<sup>b</sup> N = 12.

<sup>c</sup> N = 3.

<sup>d</sup> Significantly different than control at  $p < 0.05$ , using a two-factorial analysis of variance.

Neurotoxic signs were observed in all of the hens that received TOCP. Neither the jet engine oil test groups nor the corn oil control groups showed signs of acute, delayed neurotoxicity. One test

hen (8323-1) and one TOCP-dosed hen died during the observation period; however, neither animal showed neurotoxic symptoms prior to death.

Gross observations at necropsy showed that six of the 12 hens that were exposed to TOCP at doses from 60 to 90 mg/kg had grossly reduced skeletal muscle mass. Among hens dosed with 9082-1 oil, two had cystic ovarian lesions, one had  $0.5 \times 0.2 \times 0.2$ -cm white nodules in the oviduct mesentery, and one had a 0.6-cm diameter ulcer in the midline breast skin. One hen that was dosed with 360 mg/kg/day of 8323-1 oil had a pale liver. Pale kidneys were observed in two TOCP-dosed hens. A hen that was dosed with TOCP at 90 mg/kg/day and died spontaneously on Day 9 had atrophic myocardial and mesenteric fat and numerous ova in the abdominal cavity. A hen that was dosed with 8323-1 oil at 300 mg/kg/day had multiple yellow-to-tan, firm nodules, 0.2 to 1.0 cm in diameter, in the liver; dark red-to-purple lung parenchyma; a firm tan mass with adhesions to the liver ovary and proventriculus; multiple firm, white-to-tan 0.1 to 0.3 cm in diameter ovarian nodules, and pale kidneys.

The histopathologic diagnoses for each tissue alteration and their corresponding incidences have been listed in Table 6.1-3. The average severity scores of neural lesions are listed in Table 6.1-4. Of the two hens that died spontaneously, the one that received 300 mg/kg/day of oil 8323-1, had a cholangiocarcinoma that was a primary tumor of the gall bladder and was metastatic to the liver, an ovarian papillary adenocarcinoma, and chronic active peritonitis. These findings correlated to the gross findings. The sciatic nerve of this hen had isolated segmental demyelination that involved a single node of the nerve fiber. Histologic findings in the 90-mg/kg/day TOCP hen that died spontaneously were limited to isolated segmental demyelination of the sciatic nerve that was minimally severe.

The incidence data indicate the frequent occurrence of demyelination of the sciatic nerve in all experimental animal groups, even the controls. The average severity score for demyelination never exceeded 2.0 (slight) for any dose group. Although the incidence data indicate more frequent occurrence of axonal degeneration in the sciatic nerve of TOCP-dosed hens than in other dose groups, the difference in incidence was not significant statistically. The statistical analyses did not disclose intergroup differences in the occurrence of any histologic lesions on the basis of compound or dose-level comparisons.

#### DISCUSSION

The lesions observed in this study that may occur as a morphologic manifestation of OPIDN include demyelination, axonal and myofiber degeneration, and myositis. These lesions also may occur as minimally to mildly severe background lesions. Lesions seen in controls and most hens given the two jet engine oils were minimally severe, and were only slightly more severe in TOCP-dosed

hens. Consequently, most of the lesions observed, including neoplasms and inflammatory changes, have been considered background lesions. Clearly, the statistical analyses of histopathologic lesion incidence data or neural lesion average severity data did not reveal significant intergroup differences. The occurrence of background neural lesions in mature hens has been documented (Bickford and Sprague, 1982). Weakly pathogenic persistent viral infections may account for the background neural lesions. The frequent findings of lymphocytic inflammatory changes in tissues of hens used in this study suggested a persistent viral infection. Despite the lack of more marked neural lesions or statistically significant dose-group differences the clinical signs merit consideration in the final judgment of the OPIDN potential of the chemicals tested. Presently, no definitive quantitative correlation of the severity of neural lesions with the clinical signs of OPIDN is applied in regulatory-type testing of the OPIDN potential of chemicals.

The exact causes of death of the two hens that died spontaneously are unknown. Both the cholangiocarcinoma and peritonitis that occurred in one animal may have contributed to the demise of that animal. Surviving hens in this study, receiving five consecutive oral doses of up to 420 mg test material/kg body weight, remained neurologically asymptomatic throughout the 30-day period. Under the conditions of this study, neither of the two fluids tested can be considered neurotoxic. If human response to these engine oils parallels that of the hen, no neurotoxic hazard would be expected for military or civilian personnel involved in the manufacture, transportation, or handling of these compounds.

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TABLE 6.1-3. HISTOPATHOLOGY INCIDENCE SUMMARY

	Material:	9082				8323				TOCP				Corn Oil 1 mL/kg
		Dose (mg/kg)	420	360	300	240	420	360	300	240	90	75	60	
<b>Animals on Study:</b>														
<b>Brain<sup>a</sup></b>		4	4	4	4	4	4	4	4	4	4	4	4	12
Lymphocytic perivasculitis		4	4	4	4	4	4	4	4	4	4	4	4	12
Lymphocytic leptomeningitis		2 <sup>b</sup>	1	2	2	2	2	1	2	2	1	0	0	3
Gliosis		0	0	0	0	0	0	0	0	0	0	0	0	1
Mineralization, leptomeningeal vein		1	0	1	1	1	0	1	0	0	0	0	0	4
<b>Cervical spinal cord</b>		0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic perivasculitis		4	4	4	3	0	0	0	0	0	3	4	4	12
Demyelination		4	4	4	3	0	0	0	0	0	0	0	0	1
Axonal degeneration		1	0	0	0	0	0	0	0	0	1	1	0	0
<b>Thoracic spinal cord</b>		0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration		4	4	4	3	0	0	0	0	0	1	1	0	12
Lymphocytic perivasculitis		4	4	4	3	0	0	0	0	0	1	1	0	1
Gliosis		0	0	0	0	0	0	0	0	0	0	0	0	0
Perikaryal eosinophilic cytoplasmic granules		0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Lumbosacral spinal cord</b>		0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic perivasculitis		4	4	4	4	4	4	4	4	4	4	4	4	12
Gliosis		0	1	0	0	0	0	0	0	0	0	0	0	1
Lymphocytic leptomeningitis		0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration		0	0	0	0	0	0	0	0	0	0	0	0	0
Perikaryal eosinophilic cytoplasmic granules		0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Sciatic nerve</b>		4	4	4	4	4	4	4	4	4	3	3	3	12
Lymphocytic perineuritis		1	1	0	1	1	1	1	1	1	0	3	1	2
Demyelination		4	3	4	4	4	3	4	4	3	3	4	3	11
Inflammation, interstitial		1	0	0	0	2	1	1	0	2	1	2	1	2
Lymphocytic perivasculitis		0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration		0	0	0	0	0	0	0	0	0	0	0	0	0
Schwann cell hyperplasia		0	0	0	0	0	0	0	0	0	2	2	0	0
Lymphocytic medial arteriolitis		0	0	0	0	0	0	0	0	0	0	0	0	0

(continued)

TABLE 6.1-3. Continued

	Material:	9082				8323				TOCP			Corn Oil	
		Dose (mg/kg)	420	360	300	240	420	360	300	240	90	75	60	1 ml/kg
<i>Gastrocnemius muscle<sup>a</sup></i>		4	4	4	4	4	4	2	4	3	4	4	4	12
Subacute myositis	0 <sup>b</sup>	0	0	0	0	0	0	0	0	0	0	0	2	1
Inflammation, interstitial	2	1	0	3	4	0	0	1	0	0	0	1	0	0
Myofiber degeneration	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>Liver</i>		1	2	1	2	2	1	1	1	1	2	2	1	5
Lymphocytic aggregate, periportal	1	0	0	0	0	1	0	0	0	1	0	0	0	0
<i>Kidney</i>		0	2	1	0	2	1	1	1	1	1	2	1	1
Lymphocytic interstitial nephritis	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Lymphocytic ureteritis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Ovary</i>		2	4	3	3	4	4	2	4	3	4	4	4	8
Ovarian cyst	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Oviduct</i>		2	3	3	3	4	4	2	4	3	4	4	4	8
Chronic active salpingitis	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Lymphocytic salpingitis	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Skin</i>		2	3	3	2	4	3	1	2	2	3	1	5	5
Fibrosarcoma	0	0	1	0	0	0	0	0	0	0	0	0	0	0

<sup>a</sup>The number of animals in which the organ was examined appears in the row of data for each organ.

<sup>b</sup>The number of animals with each lesion is in the corresponding space for each lesion and treatment group.

TABLE 6.1-4. NEURAL HISTOPATHOLOGIC LESIONS AVERAGE SEVERITY SCORES

Material:	9082						8323						TOCP			Corn Oil	
	Dose (mg/kg)	420	360	300	240	420	360	300	240	90	75	60	1 mL/kg				
<b>Animals on Study:</b>																	
<b>Animals Necropsied:</b>	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	12
<b>Brain</b>	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	12
Lymphocytic perivasculitis	.5 <sup>a</sup>	.3	.5	1	.5	.5	.5	.5	.5	.7	.3	0	0	0	0	0	.3
Lymphocytic leptomeningitis	.5	0	.3	.3	.3	.3	.3	.3	.3	0	0	0	0	0	0	0	.1
Gliosis	.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.4
Mineralization, leptomeningeal vein	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Cervical spinal cord</b>	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Lymphocytic perivasculitis	.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.3
Demyelination	.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.3
Axonal degeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Thoracic spinal cord</b>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic perivasculitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gliosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Perikaryal eosinophilic cytoplasmic granules	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Lumbosacral spinal cord</b>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic perivasculitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gliosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic leptomeningitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Perikaryal eosinophilic cytoplasmic granules	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<b>Sciatic nerve</b>	3	.5	0	.8	.3	1.3	0	.3	1.7	1.3	.5	.3	.8	.5	.2	.2	.2
Lymphocytic perineuritis	1.3	.8	1.0	1.0	.8	1.3	1.3	.3	1.7	1.8	.5	.3	.5	.5	.3	.2	.2
Demyelination	1.3	0	0	.8	.3	.3	.3	0	.5	.3	0	0	0	0	0	0	0
Inflammation, interstitial	0	0	0	.8	.5	0	0	.5	0	0	0	0	0	0	0	0	0
Lymphocytic perivasculitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Axonal degeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Schwann cell hyperplasia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytic medial arteriolitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

<sup>a</sup>The average severity score of each lesion is in the corresponding space for each lesion and treatment group. The progressive scoring codes for assessing lesion severity were 1 = minimal, 2 = slight (mild), 3 = moderate, 4 = significant, and 5 = severe.

## 6.2 METABOLISM OF CYCLOTRIPHOSPHAZENE (CTP) HYDRAULIC FLUID

J.M. Gearhart, J.M. Hambel, and H.C. Higman

### ABSTRACT

Cyclotriphosphazene (CTP) is a candidate hydraulic fluid being developed by the Navy. To more efficiently plan long-term toxicity studies of this compound, a physiologically based pharmacokinetic model for CTP is under development. One piece of information necessary for model development is enzymatic metabolism of CTP. CTP was incubated for 1 min to 24 h with blood and liver tissue homogenates to determine metabolism and if the metabolites formed would chromatograph with the parent CTP hydraulic fluid. There was evidence of CTP metabolism in both homogenates as shown by the continual decrease in peak heights with longer incubation times. The greatest amount of metabolism appeared to occur in the liver, as evidenced by the appearance of at least three new chromatographic peaks that increased in height with longer periods of incubation. Incubation of CTP with blood homogenates showed less overall metabolism than liver and fewer new peaks were formed. There did appear to be a more preferential metabolism by blood of one component of CTP in comparison to the others, which may be indicative of the narrower spectrum of metabolic capabilities in blood.

### INTRODUCTION

CTP cyclic esters have been developed by the Navy as candidate hydraulic fluids. The hydraulic fluid of current interest contains 0.1% tolyltriazole (oral LD<sub>50</sub> = 675 mg/kg in rats, Huntingdon Research Center Report, 1972), an additive that inhibits copper corrosion. Toxicity studies have demonstrated that an acute oral or dermal administration of CTP was nontoxic (Kinkead and Bowers, 1985; Kinkead and Bashe, 1987). Eye and skin irritation tests proved negative, as did skin sensitization tests (Kinkead and Bowers, 1985). The hydraulic fluid was not detected in the blood or urine of rats following exposure by aerosol inhalation or dermal contact (Kinkead and Bashe, 1987). A 21-day repeated inhalation exposure in rats showed no clinical chemistry effects due to CTP exposure, but there was a significant time-dependent decrease in weight gain in the exposed vs. the sham-exposed animals (Kinkead et al., 1988). Weights of spleen (decreased), liver (increased), and testes (increased) of exposed male rats were significantly different from the controls, whereas the females had increased liver/body weight ratios for the highest dose group. Significant numbers of hyaline droplets were seen in the kidneys of CTP-exposed animals of both sexes. The lungs of the exposed groups exhibited dose-responsive increases in the number of pulmonary alveolar macrophages. A 21-day repeated dermal exposure of rabbits revealed no treatment-related effects due to CTP exposure.

In order to plan long-term toxicity studies more effectively it would be beneficial to use the predictive ability of biologically based simulation models to provide guidance in experimental design. To develop a kinetic model for CTP requires estimation of the metabolism of this compound. This initial study was conducted to gain preliminary information on *in vitro* metabolism of CTP in blood and liver whole homogenates.

#### MATERIALS AND METHODS

##### Chemicals

CTP was supplied by NMRI-TD (Batch 87-174-01). The pertinent data on this material is provided below.

##### CTP Ester

Vapor Pressure, mm Hg	0.49 (at 65 °C) 12.00 (at 149 °C)
Specific Gravity (g/mL)	1.445
Tolytriazole ( $C_7H_7N_3C$ )	
CAS No.	29385-43-1

This hydraulic fluid is a mixture of parent compound isomers including dimers, trimers, and tetramers of CTP.

All solvents and reagents were of analytical or high performance liquid chromatography grade. Sterile 0.9% sodium chloride (USP) was from Abbott Laboratories (North Chicago, IL).

##### Analytical Instrumentation

CTP was quantitated using a model 5880 Hewlett-Packard (HP) gas chromatograph (GC) equipped with an HP flame photometric detector specific for phosphorus compounds. The GC was equipped with an HP-1 crosslinked methyl silicon column. The carrier gas ( $N_2$ ) had a flow rate of 10 mL/min. Detector and injector temperatures were both 200 °C. The oven temperature was initially set at 50 °C for 2 min, then increased at a rate of 20 °C/min to a final temperature of 240 °C and maintained at this temperature for 20 min.

##### Metabolism Studies of CTP

Fischer 344 rats were obtained from Charles River Breeding Laboratories. Animals were quarantined for two weeks to allow for acclimatization and quality assurance. They were fed Purina Formulab #5008 rat chow and allowed deionized water ad libitum. Animals were sacrificed using  $CO_2$ . Blood samples were obtained from the inferior vena cava using a 10-cc heparinized syringe. Liver also was collected at this time and kept on ice until being homogenized. Liver and whole blood homogenates were made using 0.9% NaCl to yield 1:3 dilutions. Organ dilutions were homogenized with a Tissumizer (Tekmar, Cincinnati, OH) for 1 min. Stock solutions of CTP were made up in 100% denatured ethanol. Samples were incubated (37 °C) in a HaakeBuchler Evapotec vortex

evaporator for different time intervals (0, 1, 15, 60, 120, and 1440 min). After incubation, 0.5 mL of homogenate was removed at each time point and immediately extracted in screw-cap vials with 1.0 mL hexane.

## RESULTS

Gas chromatograms of CTP incubated with blood or liver homogenates show an overall decrease in peak heights with increased incubation time for all components of CTP (Figures 6.2-1 and 6.2-2). This is evident especially in blood and liver homogenates incubated for 120 min (Figures 6.2-1f and 6.2-2e).

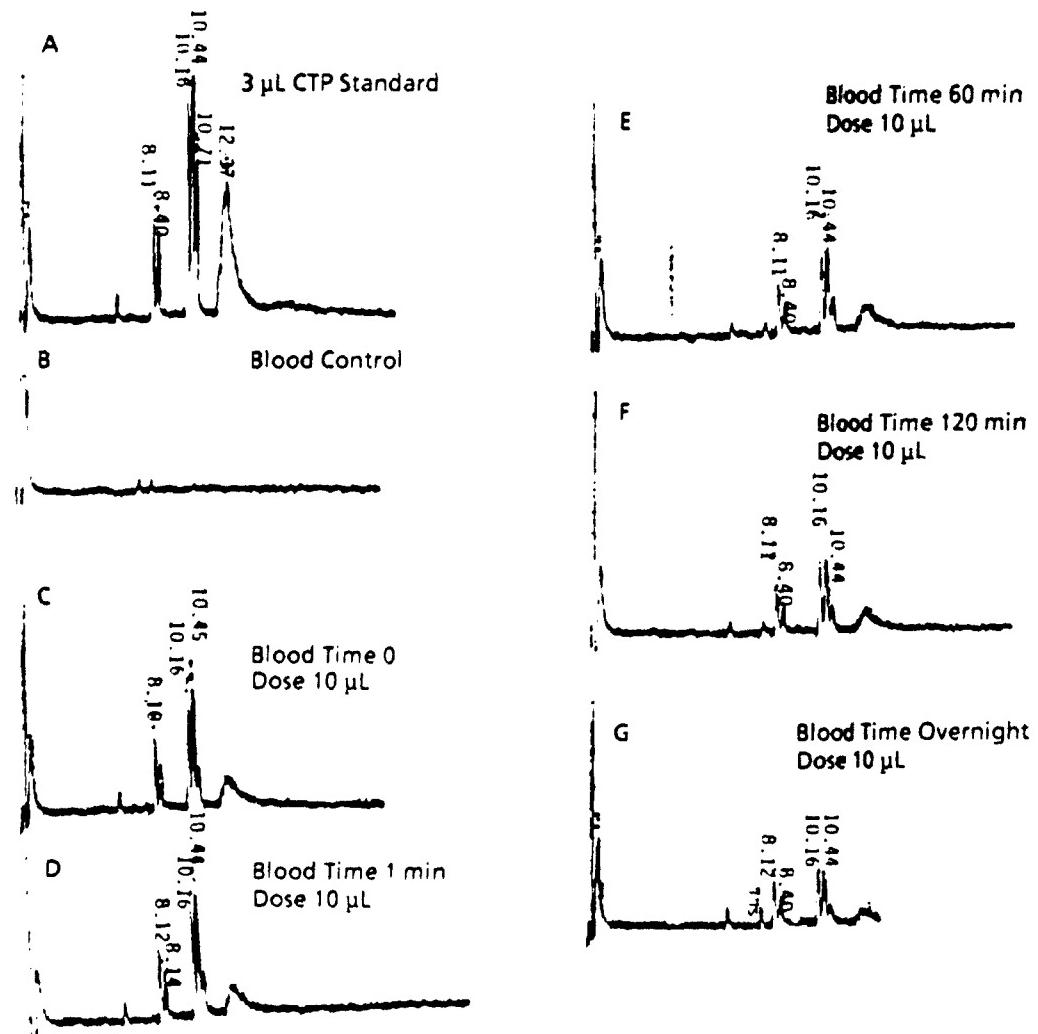
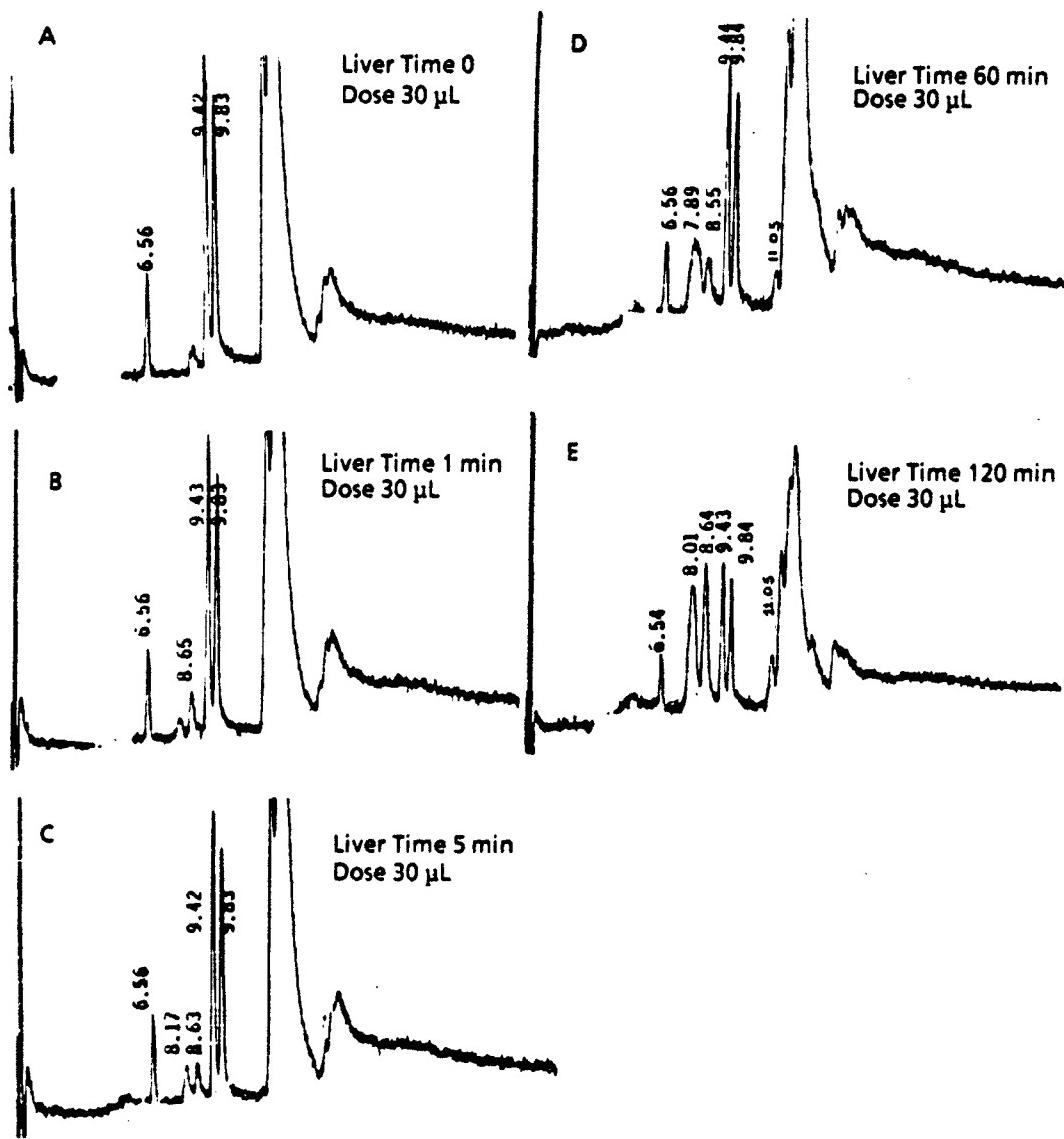


Figure 6.2-1. Gas Chromatograms of CTP Extracted from Blood after Incubation for 0 (c), 1 min (d), 60 min (e), 120 min (f), and 24 h (g). Numbers above the peaks represent the retention time of each component of CTP on the GC column.



**Figure 6.2-2. Gas Chromatograms of CTP Extracted from Liver after Incubation for 0 (a), 1 min (b), 5 min (c), 60 min (d), and 120 min (e). Numbers above the peaks represent the retention time of each component of CTP on the GC column.**

Chromatograms of CTP incubated overnight with blood homogenates indicated an alteration in the relative ratios of the different components of CTP. The peak at 10.44 is the most predominant component of CTP in all chromatograms except the sample incubated overnight. After incubation for this length of time, it became the second most predominant peak. Peaks at times 10.16, 10.44, and

10.71 also showed a greater decrease in relative concentration with time in comparison to peaks at times 8.11 and 8.40.

There was the appearance of a new peak at retention time 7.75 in the blood homogenate that was incubated overnight (Figure 6.2-1g). Evidence of this peak occurred at earlier times of incubation, but the peak was not large enough to integrate under the present chromatographic conditions.

Chromatograms of CTP incubated overnight with liver homogenates did not indicate an alteration in the relative ratios of the different components of CTP, but did indicate the production of a greater number and amount of new peaks (Figure 6.2-2e). New peaks occurred at retention times of 8.01, 8.64, and 11.05 min. The peak at time 8.01 did not chromatograph well and actually may be a composite of two or more peaks. Incubation for 120 min produced an increase in the heights of peaks 8.01 and 8.64 sufficient to equal peaks 9.43 and 9.84 of the CTP spectrum.

#### DISCUSSION

CTP was shown to be metabolized by both blood and liver tissue homogenates. Crude liver homogenates had the greatest effect on the constituent peaks of CTP by causing the greatest decrease in the overall amount of CTP and the greatest increase in the number of new peaks, or metabolites. A decrease in CTP constituent peaks and an increase in the number and heights of new peaks is indicative of CTP metabolism.

The parent ring structure of CTP is made of three nitrogen and three phosphorus molecules in a benzene-like ring (Figure 6.2-3). Attached to the phosphorus components of this parent ring are trifluoroethyl, benzyl, or phenol esters. These esters are the most likely site of enzymatic degradation of the different components of CTP. Tissue esterases are abundant in most all tissues and are known to be active especially in the liver and blood. It is likely that these new peaks are the parent ring of the different original CTP molecules with the esters removed by enzymatic degradation. Confirmation of the amount of CTP metabolites formed and the exact structure of these compounds will require further experimentation and analysis by mass spectrometry.

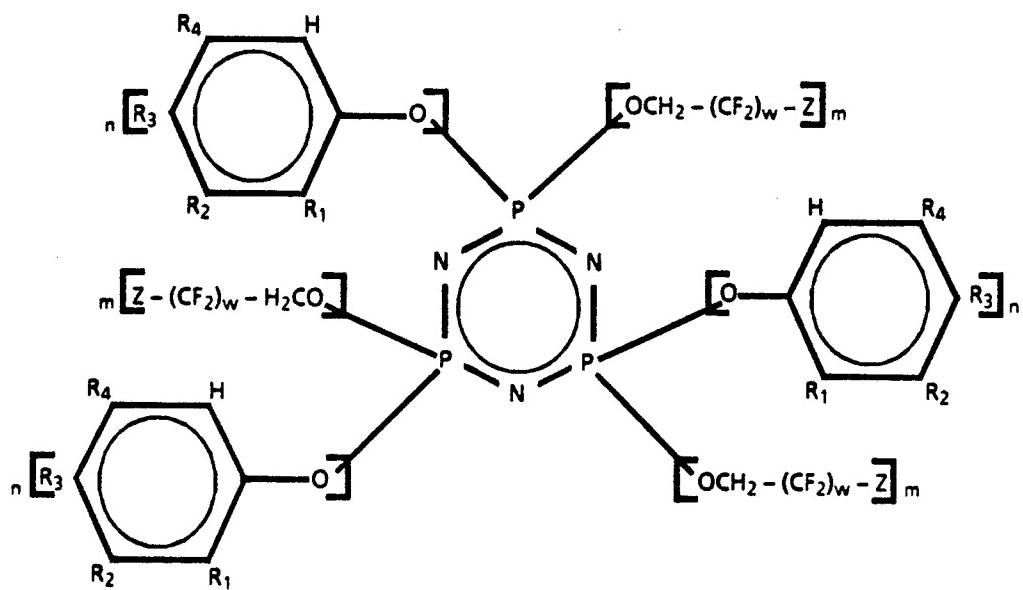
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R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>: RO<sup>-</sup>, ArO<sup>-</sup>, X--RO<sup>-</sup>, X--ArO<sup>-</sup>, X  
 Z: For H                  n, m: 0, 1, 2                  w: 1, 2, 3, ... 7  
 X: halogen

Trimer  
or  
Tetramer

Figure 6.2-3. Cyclotriphosphazene.

### **6.3 DOSE- AND SEX-SPECIFIC KINETICS OF TRIMETHYLOLPROPANE PHOSPHATE (TMP-P) AFTER DERMAL ABSORPTION**

**J.M. Gearhart, J.M. Hambel, and E.R. Kinkead**

#### **ABSTRACT**

Trimethylolpropane phosphate (TMP-P) is a thermal decomposition product of synthetic engine lubricants having a trimethylolpropane triheptanoate base. This compound is neurotoxic and belongs to a class of compounds commonly referred to as bicyclophosphorus esters (BCPEs). Studies indicate that the specific target of TMP-P is the central nervous system where the compound binds to the gamma-aminobutyric acid (GABA) receptor and prevents GABA-mediated neurotransmission. The kinetics of TMP-P in blood were determined in male and female Fischer 344 (F-344) rats after a dermal dose of pyrolyzed lubricant containing 30 or 60 mg TMP-P/kg body weight. The blood levels of TMP-P after the 60-mg/kg treatment were significantly greater ( $p < 0.009$ ) in the males than the females, and the blood concentrations of TMP-P in males after 60 mg TMP-P/kg body weight also were different significantly ( $p < 0.008$ ) from the 30-mg/kg treatment. A significant ( $p < 0.048$ ) interaction between sex and dose also was found. The kinetics of TMP-P in the blood of the females was not dose dependent at these two treatment levels. Skin thickness was measured in each sex to determine if it could be a factor in the sex-specific kinetics of TMP-P. The males had a skin thickness of 1.04 mm and the females had a skin thickness of 0.78 mm, suggesting that skin thickness is not a significant cause of the difference of TMP-P kinetics between the sexes.

#### **INTRODUCTION**

Synthetic aircraft engine lubricants with a trimethylolpropane triheptanoate base (MIL-L-23699) are used extensively in commercial and military jet propulsion systems. During pyrolysis these lubricants have been shown to form a neurotoxic compound, TMP-P. TMP-P belongs to a class of compounds commonly referred to as BCPEs. The specific target of BCPEs is the central nervous system where the compounds bind to the GABA receptors and prevent GABA-mediated neurotransmission (Bowery et al., 1976).

Acute LD<sub>50</sub>s of TMP-P (oral, dermal, and intraperitoneal [ip]) in various species of animals have been reported by Kimmerle et al. (1976). The results of this study showed that the acute toxicity values of TMP-P were similar among all species tested (mouse, rat, guinea pig, hamster, rabbit, cat, dog, chicken, and quail). The chicken was the least sensitive to the compound. No sex-specific differences were noted in any species studied. Wyman et al. (1987) added TMP-P to non-pyrolyzed lubricant to determine the minimum level required to produce neurotoxic effects in rats and mice

when dosed ip. The lowest dose that produced convulsions was 0.5 mg/kg for rats and 0.8 mg/kg for mice. The sex of the animals was not reported.

The acute dermal toxicities of TMP-P, administered in pyrolyzed MIL-L-23699 lubricant samples to male and female B6C3F<sub>1</sub> mice, were approximately 50 and 100 mg/kg, respectively (Porvaznik et al., 1988). The lethal effects were prevented if the exposure sites were washed and dried within 5 min following application. In the report by Kimmerle (1976) it was determined that the toxicity of TMP-P was not gender-related; however, the compound was prepared in an emulsion (Cremophor EL) and remained on the skin for 24 h. The twofold greater LD<sub>50</sub> of TMP-P in female mice suggests that the rate of percutaneous absorption may be different in females than in males when the contaminant is applied in non-pyrolyzed lubricant.

The rat was selected as the test species for this study because it is of sufficient size that serial blood samples (80 µL per sample) can be drawn without jeopardizing the health and well-being of the animal. The numbers of animals per group have been selected to facilitate evaluation of the statistical significance of the results.

A toxicity hazard may exist for personnel exposed to the combustion products of pyrolyzed synthetic aircraft engine lubricants with an MIL-L-23699 base following aircraft engine fires. This study was designed to determine whether a gender difference exists in the dermal uptake of pyrolyzed MIL-L-23699 lubricating oil.

## MATERIALS AND METHODS

### Chemicals

TMP-P, batch No. 5323-1, was synthesized by Wright State University through contract with NMRI/TD. Pyrolysate, Exxon 2380, was obtained through NMRI/TD as pyrolyzed MIL-L-23699 lubricating oil. All solvents and reagents were of analytical or high performance liquid chromatography (HPLC) grade.

### Instrumentation

TMP-P was quantitated using a model 5880 Hewlett-Packard gas chromatograph (GC) equipped with a Hewlett-Packard flame photometric detector specific for phosphorus compounds. The GC was equipped with a Hewlett-Packard HP-1 (crosslinked methyl silicone) 10 M x 0.53 mm x 0.88 µm 100% dimethyl polysiloxane, nonpolar column. The carrier gas (N<sub>2</sub>) had a flow rate of 10 mL/min. Detector and injector temperatures were both 200 °C. The oven temperature was initially set at 50 °C for 2 min, then increased to a final temperature of 240 °C at a rate of 20 °C/min. TMP-P eluted at a retention time of 9.60 min. External standards, which ranged from 10 ng/mL to 10 µg/mL, were run with biological samples (3 µL injections), and compared to standard

curves. The extraction efficiency of TMP-P from tissues was 70% using acetone or ethyl acetate as the solvents of choice. The lower limit of detection for TMP-P was 10 ng/mL.

#### **Pharmacokinetic Dermal Studies**

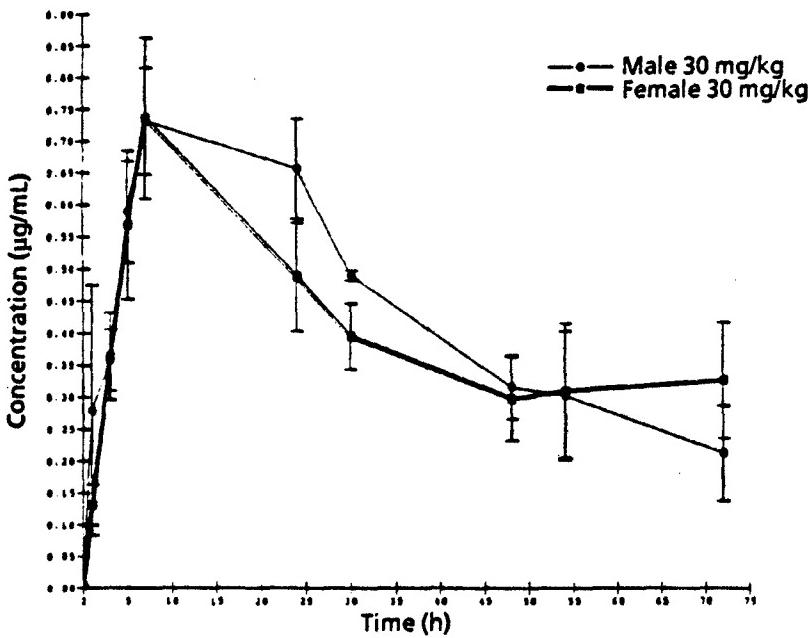
F-344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY) and randomized by weight (male rats 200 to 350 g and female rats 150 to 250 g). Upon receipt, the animals were housed for quarantine and quality assurance purposes. Animals were identified by toe clip. During exposure the animals were housed in metabolism cages and fed powdered Purina Formulab #5008 rat chow and deionized water ad libitum. One day prior to exposure, a 3 cm x 3 cm area of dorsal surface was clipped and observed for abrasions. If abrasions were noted the animal was removed from the experiment and another was used. Two hours prior to exposure, scintillation vial caps were applied to the clipped area with Loctite Super Bonder (Loctite Corp., Newington, CT) instant adhesive. Animals were dosed with TMP-P spiked pyrolysate to give final dose concentrations of 30 or 60 mg TMP-P/kg body weight. Blood samples were taken from the lateral tail vein of all animals at 1, 3, 5, 7, 24, 30, 48, 54, and 72 h. Animals were sacrificed in groups of five at 30 and 72 h after application of the pyrolysate.

**Skin Thickness.** Each animal's skin thickness was measured at sacrifice with a Dyer Oditest gauge (Dyer Co., Lancaster, Pa). The site of skin thickness measurement was made adjacent to the site of the liquid scintillation cap application.

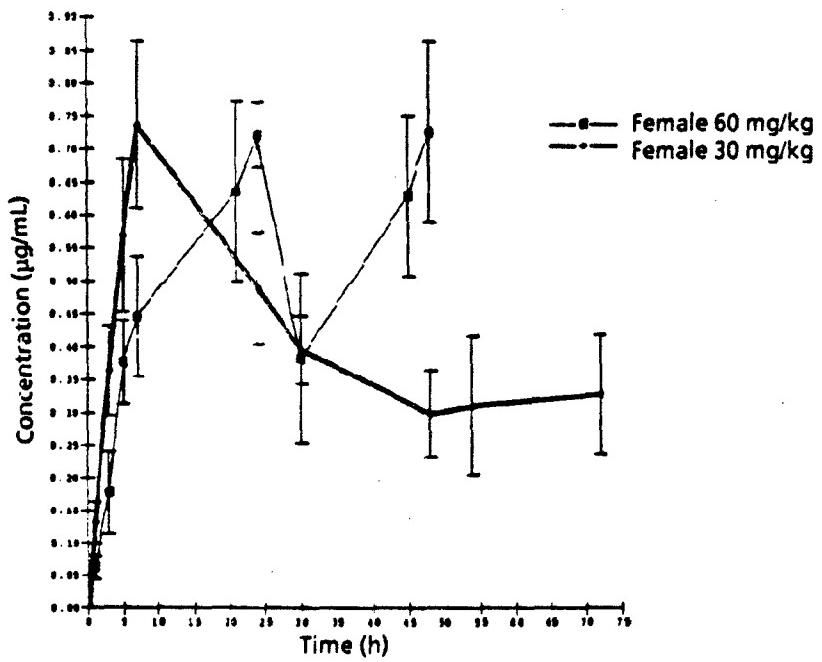
**Statistical Analysis.** The blood kinetics data were analyzed for sex and dose differences by a two-factorial analysis of variance with repeated measures. The skin thickness measurements were analyzed by a two-factorial analysis of variance.

#### **RESULTS**

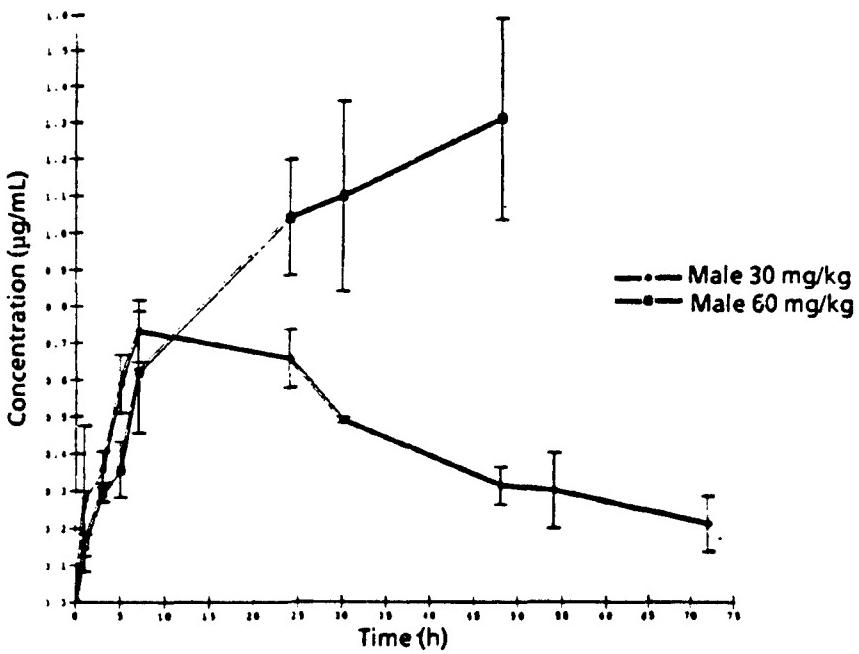
There was no difference in the blood kinetics of TMP-P between male and female F-344 rats after a 30-mg/kg dermal dose of TMP-P in pyrolyzed lubricant (Figure 6.3-1). There also was no difference in the blood kinetics between the two female groups dosed at 30 vs. 60 mg/kg (Figure 6.3-2). A significant difference in the blood concentration of TMP-P over time between males dosed with 30 mg/kg TMP-P was found when compared to those dosed with 60 mg/kg (Figure 6.3-3). This also was true for the two sexes dosed with 60 mg/kg TMP-P (Figure 6.3-4). The initial rate of TMP-P uptake into the blood was very similar for all dose and sex groups (Figure 6.3-5) through the 7-h time period. The major notable difference between all exposure groups was the continued rise in blood concentration in the 60-mg/kg male group (Figure 6.3-5), whose blood levels of TMP-P continued to rise at 48 h after dosing, at which time the animals were sacrificed to avoid the potential lethal effects of TMP-P.



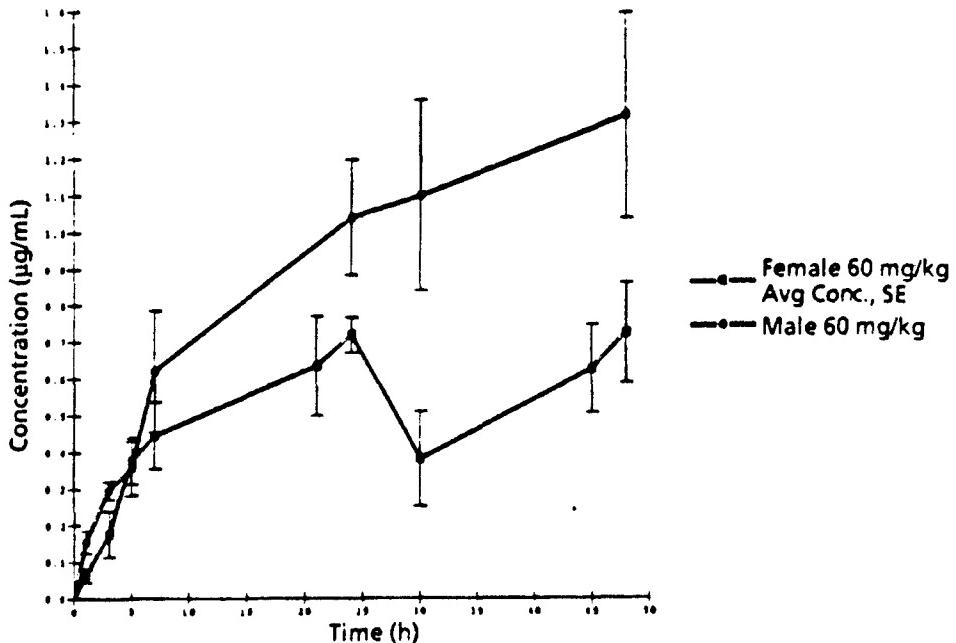
**Figure 6.3-1.** Concentration of TMP-P in Male Versus Female F-344 Rats after Dermal Exposure to 30 mg TMP-P/kg Body Weight. Each datum point represents the group mean, whereas the error bars represent  $\pm$  one standard error of the mean.



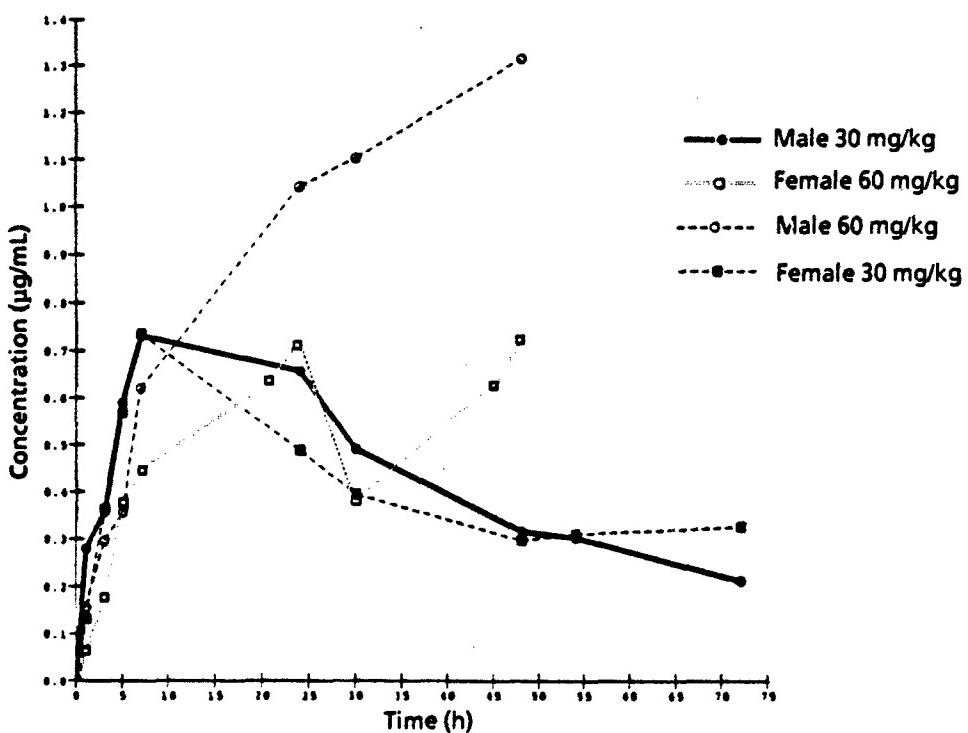
**Figure 6.3-2.** Concentration of TMP-P in Two Groups of Female F-344 Rats after Dermal Exposure to 30 or 60 mg TMP-P/kg Body Weight. Each datum point represents the group mean, whereas the error bars represent  $\pm$  one standard error of the mean.



**Figure 6.3-3.** Concentration of TMP-P in Two Groups of Male F-344 Rats after Dermal Exposure to 30 or 60 mg TMP-P/kg Body Weight. Each datum point represents the group mean, whereas the error bars represent  $\pm$  one standard error of the mean.



**Figure 6.3-4.** Concentration of TMP-P in Male Versus Female F-344 Rats after Dermal Exposure to 30 or 60 mg TMP-P/kg Body Weight. Each datum point represents the group mean, whereas the error bars represent  $\pm$  one standard error of the mean.



**Figure 6.3-5.** Concentration of TMP-P in Male Versus Female F-344 Rats after Dermal Exposure to 60 mg TMP-P/kg Body Weight. Each datum point represents the mean, whereas the error bars represent  $\pm$  one standard deviation of the mean.

The skin thickness of males (1.0425 mm) was significantly greater than that of females (0.780) ( $p < 0.0001$ ). This overall difference also was significant at the 30-h ( $p < 0.017$ ) and 72-h ( $p < 0.01$ ) sacrifice time points.

#### DISCUSSION

This study was conducted to determine if the lower LD<sub>50</sub> dermal dose of TMP-P in male mice (50 mg TMP-P/kg) vs. female mice (100 mg TMP-P/kg) determined by Porvaznik (1988) could be exhibited by pharmacokinetic differences between the two sexes and if these differences could be related to differences in skin thickness. The sex difference in LD<sub>50</sub>s was born out in the blood kinetics of male vs. female rats (Figure 6.3-5), when the 60-mg TMP-P/kg exposed males achieved a much higher blood level of TMP-P than the females exposed to the same dose (Figure 6.3-3). The reasons for the differences in blood kinetics after dermal dosing with TMP-P between the two sexes cannot be explained by skin thickness because the males actually had much thicker skin than the females. It is possible that the sex differences in toxicity and TMP-P blood kinetics are due to metabolism by the skin, an organ that is recognized as having metabolic capabilities (Noonan and Wester, 1983).

differences in TMP-P's tissue affinities between the different sexes, or clearance differences between the two sexes, related to overall metabolism or excretion of TMP-P.

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## **6.4 DESIGN, PERFORMANCE, AND FLUID MECHANICS OF A SMALL ANIMAL, WHOLE-BODY INHALATION EXPOSURE CHAMBER**

**E.C. Kimmel and K.L. Yerkes**

### **ABSTRACT**

A 0.7-m<sup>3</sup> inhalation exposure chamber was designed for continuous, whole-body exposure of up to 64 small laboratory rodents to a variety of airborne toxicants using either single- or multiple-tier configuration. Though similar in design to conventional exposure chambers currently in use, several design modifications were incorporated to improve chamber performance with regard to both animal maintenance and toxicant distribution within the chamber. Several approaches were taken to assess chamber performance characteristics. Repeated, random-order, serial sampling of 27 discrete loci within the exposure volume was used to determine both spatial and temporal distribution of polydisperse sodium chloride aerosols (1.3 µm mass median aerodynamic diameter, 1.65 µg). The collective spatial and temporal deviation of aerosol concentration within the exposure volume as a whole ranged from 3.5 to 5.2% as a function of single- vs. multiple-tier configuration. Application of a mathematical model of mixing characteristics in dynamic flow reaction vessels demonstrated that the effective mixing volume in the chamber varied from 50 to 65% of total chamber volume depending upon configuration. Computational fluid mechanics methods were used to model flow structure within the exposure volume and remarkable correlation between predicted areas of flow stasis within the chamber and observed focal points of significant deviation of aerosol concentration were found.

### **INTRODUCTION**

Uniform distribution of the test material throughout an inhalation exposure apparatus is desirable to minimize nonbiologically caused variation of the inhaled dose. However, this often does not prevail; therefore, it is important to characterize distribution of test material within an exposure chamber to determine the impact of inhomogeneities of exposure, if any, on animal response to the test material. Also, knowledge of the chamber distribution characteristics is necessary for the selection of an optimal representative sampling point for routine measurements taken during inhalation exposures.

Several approaches have been taken toward analyzing exposure chamber performance. These include (1) flow visualization techniques (Moss et al., 1982) and (2) quantitative determinations of the rate of dispersion and the distribution of vapors and aerosols (Hemenway et al., 1982; Yeh et al., 1986). For the present investigation, several methods were chosen to analyze the performance of a recently designed exposure chamber in use at the Toxic Hazards Research Unit (THRU) at Wright-

Patterson Air Force Base, OH. First, a "snapshot" of test material distribution in the chamber was obtained by analysis of the overall and local variations of aerosol concentration measured at several discrete locations within the chamber. Concentration and differences in the magnitude of local variation of concentration were analyzed to determine the combined temporal and spatial variation within the chamber and to identify localized abnormalities of aerosol distribution and variability. Sequential measurements at a central reference point were used to isolate the spatial component of the combined variation (Carpenter et al., 1987). Second, chamber integrity (leakage) was determined by a modification of the method of Mokler and White (1983). Third, chamber mixing characteristics were determined using the method of Cholette and Cloutier (1959). Fourth, numerical solutions of laminar Navier-Stokes equations (Yerkes, 1990) were used to model flow structure within the chamber.

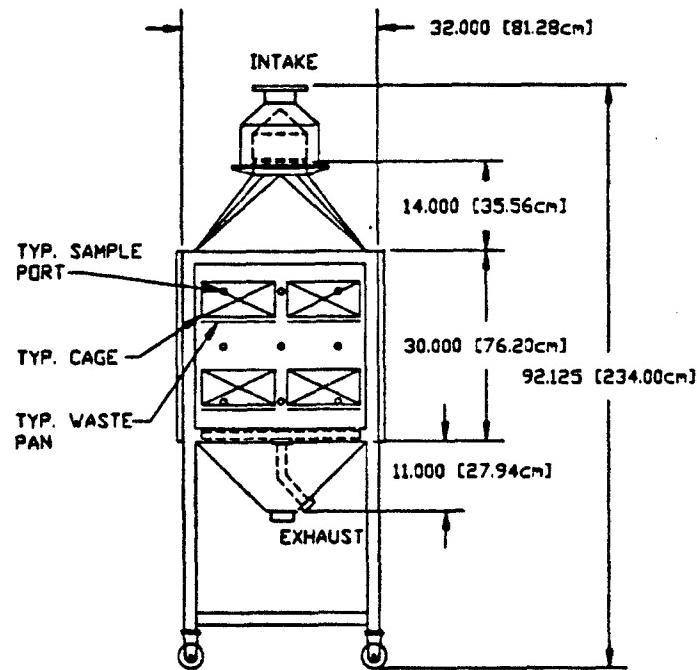
## **METHODS AND MATERIALS**

### ***Design and Operation***

The THRU chamber is a slightly larger (690 L), modified version of the 27-in. vertical flow exposure chamber described by Hinnens and associates (1968) (Figure 6.4-1). The chamber is exhausted through a distributive manifold similar to that described by Carpenter and Beethe (1981) and has an annular orifice, continuous opposed jet, and inlet geometry to enhance test material mixing and to minimize rotational and reflux flow. Chamber design allowed the use of heat-tempered plate glass for two side walls and the chamber door. The rear wall of the chamber as well as the plenums were fabricated out of stainless steel plate. For this investigation, the chamber was configured in three different manners, described in Table 6.4-1. For normal operation, two tiers of four (two per tier) horizontal cage units with excreta catch pans were suspended axisymmetrically about the vertical axis of the exposure volume. A 10-cm gap was left between the cage units themselves and between cage units and chamber walls on each tier. Exposure capacity was either 64 mice or 32 rats. The chamber was operated at flow rates of 162.2 to 170.2 L/min at 1.03 to 1.57 mm Hg subambient pressure maintaining 14.1 to 14.8 chamber volume changes per hour.

### ***Test Material Generation and Sampling***

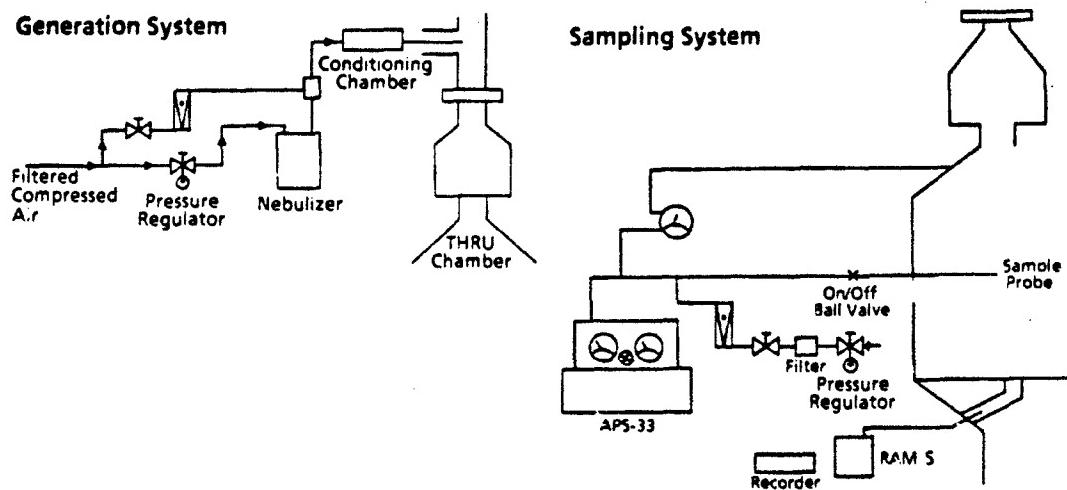
Polydisperse NaCl aerosols were generated using a modified, large reservoir Retec compressed-air nebulizer (Model X-70/N, Cavitron Corp., Portland, OR). Prior to countercurrent injection into the chamber inlet air stream, the nebulizer output was passed through a conditioning vessel maintained at 98.8 °C in which a 10-mCi  $^{85}\text{Kr}$  deionizer was located (Figure 6.4-2). Mass concentration and aerosol size distribution data for the test atmospheres are given in Table 6.4-2.



**Figure 6.4-1. Cross Section of the THRU Small Animal, Whole-Body Inhalation Exposure Chamber.**  
Dimensions in inches (centimeters).

**TABLE 6.4-1. CHAMBER TEST CONFIGURATION**

Configuration 1	Empty
Configuration 2	Two tiers of 4 cages (2/tier) with excreta pans
Configuration 3	Cages and pans with full complement of animals (32 rats)



**Figure 6.4-2. Schematic Representations of the Aerosol Generation and Sampling Systems.**

TABLE 6.4-2. CHAMBER CONCENTRATION AND AEROSOL SIZE DISTRIBUTION

	Concentration (mg/m <sup>3</sup> )	MMAD (μm)	$\sigma_g$
Configuration 1	5.76 ± 0.354	1.39 ± 0.037	1.66 ± 0.025
Configuration 2	5.78 ± 0.110	1.28 ± 0.018	1.61 ± 0.011
Configuration 3	4.17 ± 0.241	1.35 ± 0.023	1.61 ± 0.012

All values are mean ± SD.

Data are mean of mean values for five trials.

MMAD = mass median aerospace diameter.

Nine sampling ports located in the rear wall were fitted with 0.64-cm diameter stainless steel probes that could be positioned at various depths into the exposure volume. The center port also was fitted with a reference probe fixed in the exact center of the exposure volume. The exposure volume was divided into 27 identical 12.37-L cuboidal cells (Figure 6.4-3) and the center of each cell served as the sampling loci (Figure 6.4-4). In a trial run, each locus was sampled in random order and 16 reference point samples were interleaved between loci samples so that at least every third sample taken was from the reference point. Five trials were made for each of the three chamber test configurations. Aerosol concentration and size distribution were measured with an aerodynamic particle sizer (APS Model 338 with a 3302 100:1 diluter, TSI, Inc., St. Paul, MN). Each sample was taken at 1 L/min for 2 min. The time between samples was 0.5 min. Concentration at the chamber exhaust port was measured continuously with a RAM-S (MIE, Inc., Bedford, MA) optical aerosol monitor (Figure 6.4-2).

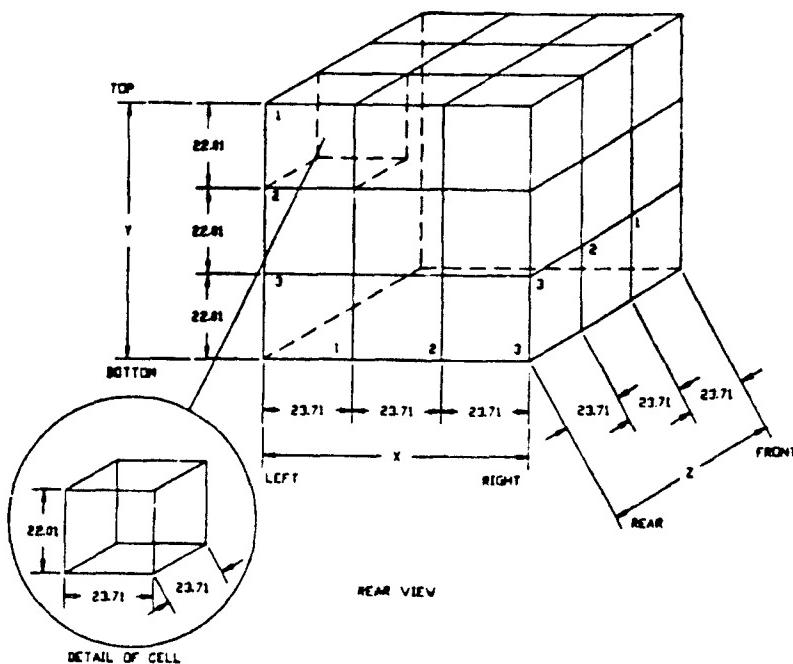
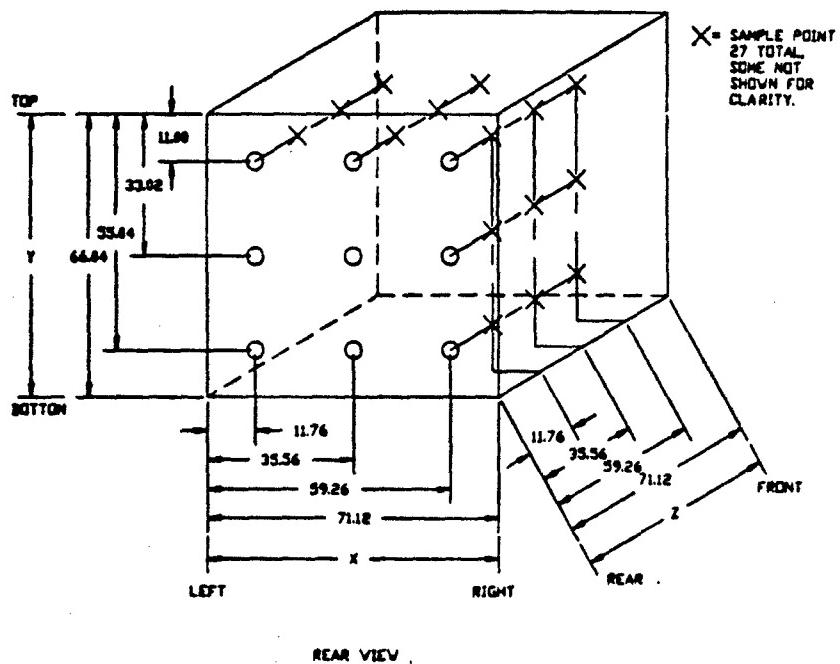


Figure 6.4-3. Diagram of 27 Sampling Cells within Chamber Exposure Volume with Coordinate Designation and Dimensions. All dimensions in centimeters.



**Figure 6.4-4.** Location of 27 Sampling Points within Chamber Exposure Volume. All dimensions in centimeters.

#### Statistics

Comparisons of intercellular differences in concentration and variability were made using either Tukey's Studentized Range Method or Ryan-Einot, Gabriel, Welsch factorial analysis of variance, where appropriate.

#### RESULTS

##### Chamber Leak Rate

The exposure chamber was found to be essentially leak free. The calculated leak rate was  $1.28 \times 10^{-7}$  L/min corresponding to  $8.8 \times 10^{-3}\%$  of the chamber volume per minute and  $1.9 \times 10^{-8}\%$  of the chamber operating flow rate.

##### Mixing Characteristics

Data for the time course of decay of the chamber steady-state aerosol concentration (after generator shutdown) were fitted to Equation 1 and plotted semilogarithmically for each of the test configurations (Figures 6.4-5 through 6.4-7).

$$C/C_0 = e^{-Qt/V} \quad (1)$$

where

- C = concentration at time t,
- $C_0$  = steady-state concentration,
- Q = chamber flow rate,
- V = chamber volume.

From these plots the fractions of chamber volume that were either dead space or well mixed with incoming air or some of the chamber exhaust flow that was generated by piston (plug) flow of incoming air were calculated (Table 6.4-3).

#### Spatial and Temporal Variation

Combined spatial and temporal variation of average chamber concentration ranged from 3.5 to 5.2% depending on chamber configuration. On the average, spatial variation accounted for 85% of the total. Relative spatial vs. temporal contribution to total variation, for all configurations, is shown in Table 6.4-4.

No intercellular differences in concentration were found in the empty chamber (Configuration 1). In Configuration 2, (Figure 6.4-8) significant differences of concentration between individual cells were found. Concentration in a block of three cells at the top left side (viewed from the rear) of the chamber was significantly higher than all other cells. Concentration in four cells located just below the upper tier of pans (middle layer of the chamber) was significantly lower than all other cells. Concentration in a single cell in the bottom layer of the exposure volume was significantly lower than all other cells. With animals in the chamber (Configuration 3 - Figure 6.4-9) no cellular differences in concentration were observed in the top or bottom layers of the exposure volume. However, as in Configuration 2, the same four cells in the middle layer had concentrations significantly lower than all other cells.

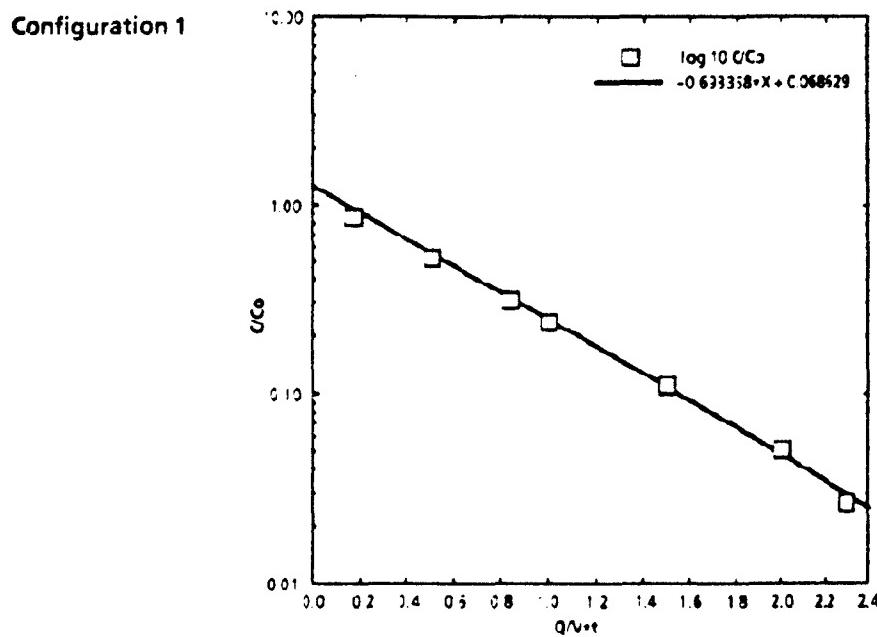
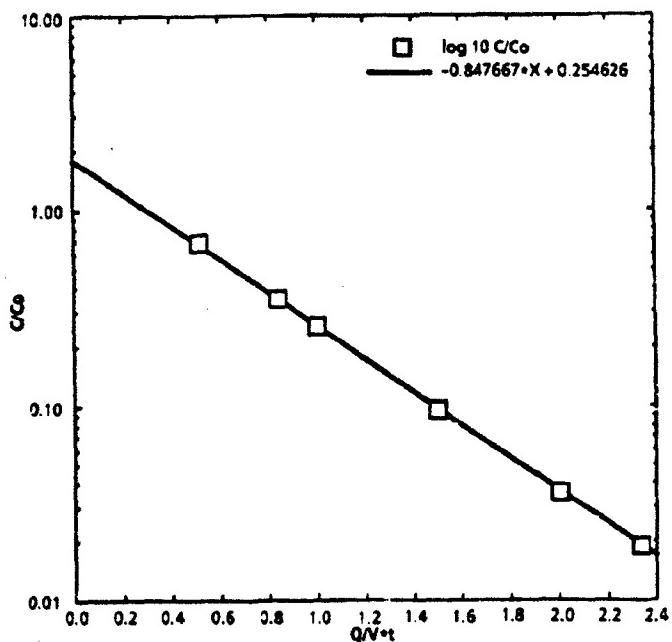


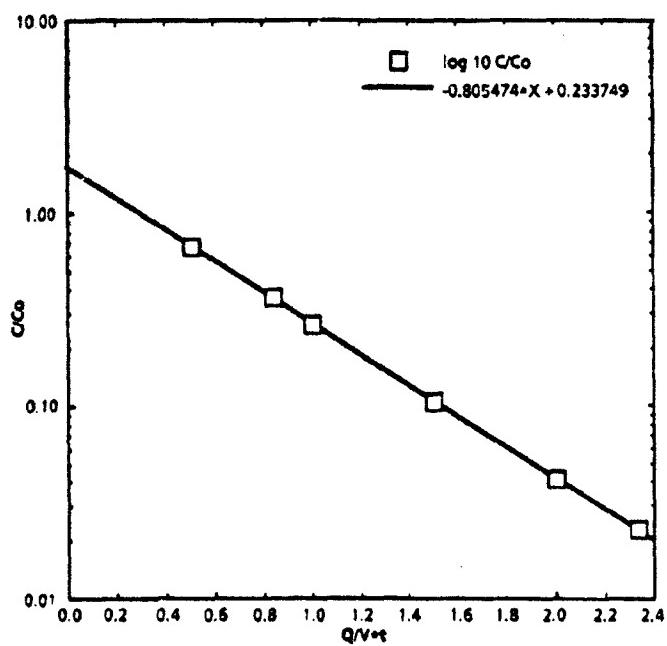
Figure 6.4-5. Chamber Concentration Decay Curve for Configuration 1 (empty chamber) after Generator Shutdown. Data points are mean of five trials.  $C$  = concentration at time  $t$ ,  $C_0$  = steady-state concentration,  $Q$  = chamber flow rate, and  $V$  = chamber volume.

**Configuration 2**



**Figure 6.4-6.** Chamber Concentration Decay Curve for Configuration 2 (cages and excreta pans) after Generator Shutdown. Data points are mean of five trials. C = concentration at time t, C<sub>0</sub> = steady-state concentration, Q = chamber flow rate, and V = chamber volume.

**Configuration 3**



**Figure 6.4-7.** Chamber Concentration Decay Curve for Configuration 3 (cages, pans, and animals) after Generator Shutdown. Data points are mean of five trials. C = concentration at time t, C<sub>0</sub> = steady-state concentration, Q = chamber flow rate, and V = chamber volume.

TABLE 6.4-3. CHAMBER MIXING CHARACTERISTICS

	Piston Flow Volume	Well-Mixed Volume	Dead Space Volume
Configuration 1	11.7	64.7	23.6
Configuration 2	27.2	51.3	21.5
Configuration 3	29.0	53.9	17.1

All values are percentages of total chamber volume.

TABLE 6.4-4. CHAMBER SPATIAL AND TEMPORAL VARIATION OF CONCENTRATION

	Total	Spatial	Temporal	Temporal % of Total
Configuration 1	$3.54 \pm 0.526$	$2.76 \pm 0.529$	$0.78 \pm 0.121$	22.0
Configuration 2	$5.24 \pm 0.551$	$4.52 \pm 0.353$	$0.71 \pm 0.509$	13.6
Configuration 3	$4.25 \pm 1.029$	$3.82 \pm 0.338$	$0.43 \pm 0.111$	10.1

All values except last column are mean  $\pm$  SD %.  
Data are mean of mean values for five trials.

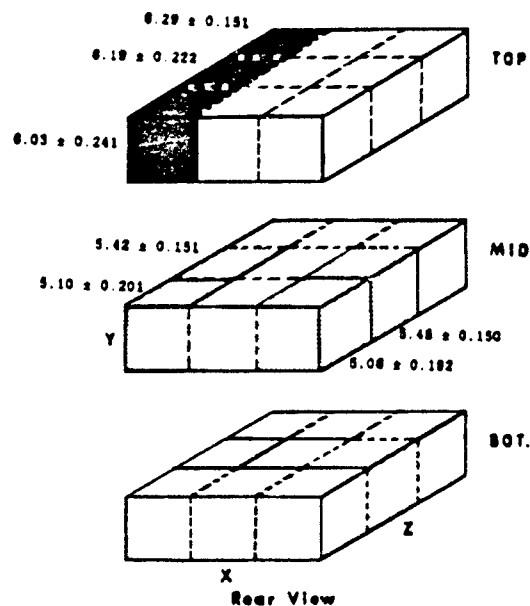
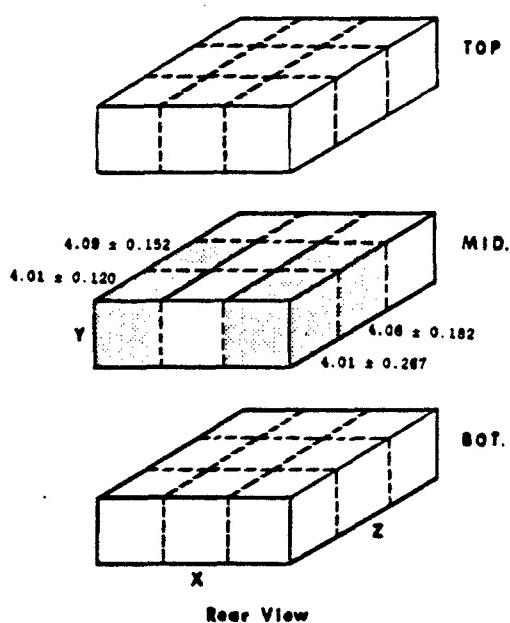


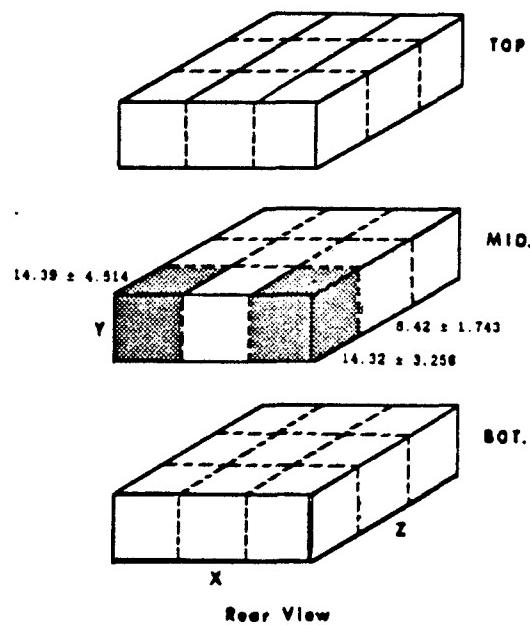
Figure 6.4-8. Configuration 2 (cages and excreta pans) Cellular Variation of Concentration. Data are mean  $\pm$  SD of mean values of five trials. Dark-shaded cells have significantly higher concentrations than all other cells at  $p \leq 0.05$ . Light-shaded cells are significantly lower than all other cells at  $p \leq 0.05$ .



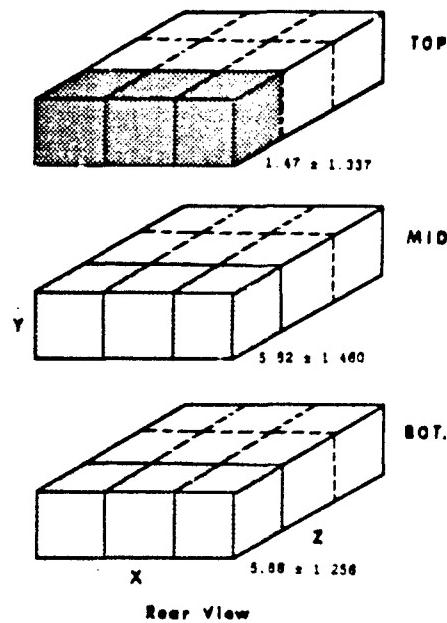
**Figure 6.4-9. Configuration 3 (cages, pans, and animals) Cellular Variation of Concentration.** Data are mean  $\pm$  SD of mean values of five trials. Shaded cells have significantly lower concentrations than all other cells at  $p \leq 0.05$ .

Differences of intercellular variability (relative magnitude of the variation of cellular concentration) were not observed in the empty chamber (Configuration 1). In Configuration 2 (Figure 6.4-10), two cells, located at the rear and to the sides of the middle layer, were significantly more variable than all other cells. One other cell in this layer was significantly more variable than all but 5 of the other 27 cells. The addition of animals to the chamber (Configuration 3 - Figure 6.4-11) changed the pattern of variability in the chamber. All nine cells located next to the metal rear wall of the chamber were found to have significant differences of variability. A block of three cells in the top layer of the exposure volume was significantly less variable than all other cells, whereas the corresponding blocks of three cells each in the middle and bottom layers of the exposure volume were significantly more variable than all other cells.

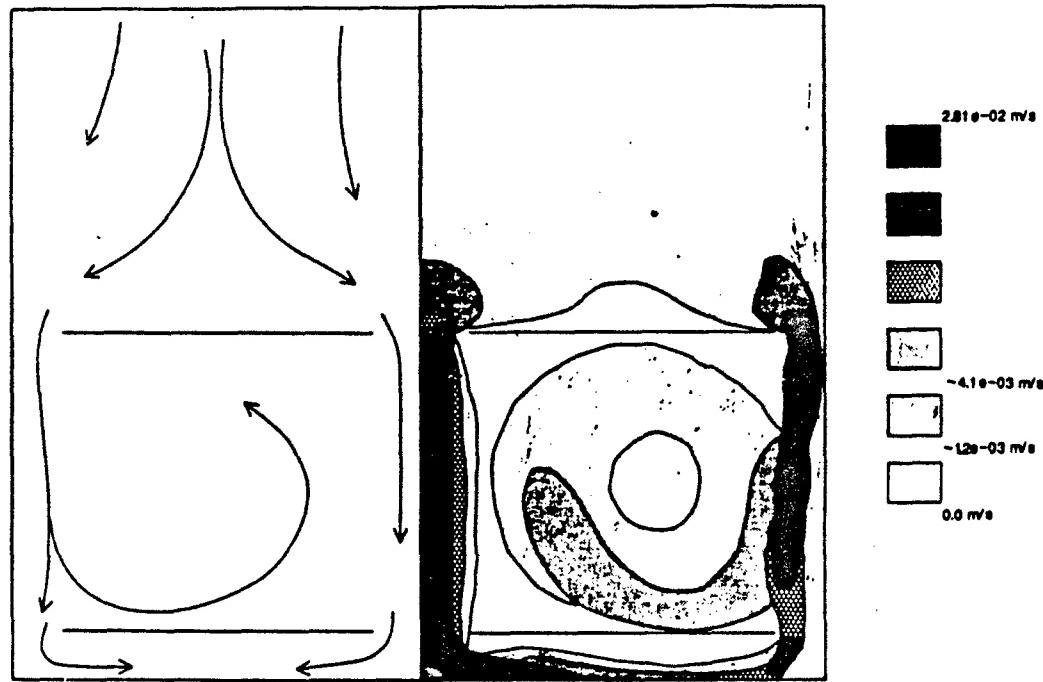
A numerical model of the flow structure in the chamber (Figure 6.4-12) showed that the excreta pans acted as baffles, causing increases in velocity (jetting) of flow down the middle of and next to the walls of the chamber. Two vortices formed just below the upper tier of pans.



**Figure 6.4-10. Configuration 2 (cages and excreta pans) Relative Cellular Spatial Variation.** Data are mean  $\pm$  SD of mean values for five trials. Dark-shaded cells are significantly more variable than all other cells at  $p \leq 0.05$ . Light-shaded cells are significantly more variable than the remaining cells (unshaded) at  $p \leq 0.05$ .



**Figure 6.4-11. Configuration 3 (cages, pans, and animals) Relative Cellular Spatial Variation.** Data are mean  $\pm$  SD of mean values for five trials. Dark-shaded cells are significantly less variable than all other cells at  $p \leq 0.05$ . Light-shaded cells are significantly more variable than all other cells at  $p \leq 0.05$ .



**Figure 6.4-12. Two-Dimensional Numerical Simulation of Chamber Flow Pattern and Velocity Magnitude. From Yerkes (1990).**

#### DISCUSSION

Overall variation of concentration in the chamber (3.5 to 5.2%, depending on configuration) was deemed acceptable and compared well with exposure chambers of comparable design. Excreta pans, acting as baffles, had effects on overall chamber performance. Likewise, local anomalies of concentration and variability partially attributed to the effects of these pans. Jetting, caused by the pans, was most likely responsible for the nearly twofold increase in piston flow, as well as the decrease of well-mixed volume. The pans also, most likely, were responsible for the increase in spatial component contribution to total chamber variation. The stagnant flow zones developed below the upper tier of pans were deemed responsible for the localized spaces of low concentration and high variability.

Inclusion of animals in the chamber also apparently affected chamber distribution characteristics. Animals, acting as heat sources, may have introduced convection currents into the chamber flow structure (Yerkes, 1990). Local deviations of concentration in the top layer of the exposure volume, which occurred without animals, were not observed. It is speculated that convection currents in this layer aided mixing, hence, a more uniform distribution of aerosol in this layer. However, these currents were not sufficient to ameliorate the effects on concentration and variability caused by the pans on the middle layer of the exposure volume. With animals in the

chamber, the effects of different materials of chamber construction and the chamber flow structure may have come into play. The metal rear wall of the chamber was more thermoconductive than the glass of the three other walls. Heat transfer, with accompanying convection currents, may have been responsible for the deviations of variability found only in all cells located next to the metal wall.

The presence of consistent, localized perturbations or concentration and variability in the chamber indicate that, if these phenomena cannot be eliminated, their effects should be accounted for in the final analysis of animal response to test material exposure.

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## 6.5 THERMAL EFFECTS IN WHOLE-BODY INHALATION EXPOSURE CHAMBERS

K.L. Yerkes

### ABSTRACT

An initial investigation to determine the effects of mixed convection on the flow structure of whole-body inhalation exposure chambers for both "aided" and "opposed" buoyant forces has been completed. Experimental results are reported for momentum boundary layer thickness and temperature distribution within the chamber. Numerical results and experimental data were obtained for comparison. Two- and three-dimensional transient numerical models were formulated using a fully implicit finite-difference scheme of the laminar Navier-Stokes equations. The inlet and wall temperatures were held constant while maintaining a uniform inlet velocity. Two tiers of four (two per tier) horizontal cage pans or baffles were located axisymmetrically about the vertical axis with the inlet at the top and exhaust at the base of the chamber. The difference between the inlet and wall temperatures ranged between  $-1.0^{\circ}\text{C}$  to  $3.3^{\circ}\text{C}$  with a Prandtl number,  $\text{Pr} = 0.7$ , inlet Reynolds number,  $\text{Re} = 32$  and 235, and inlet Rayleigh number,  $\text{Ra} = 0.6.8 \times 10^7$ . It was concluded that the flow patterns within these chambers are predominantly buoyant in nature with asymmetric behavior. The development of the flow structure was found to be sensitive to small variations in the temperature difference between the inlet and wall.

### NOMENCLATURE

#### English letter symbols

$A_1$	aspect ratio, $L/H$
$A_2$	aspect ratio, $L/D$
$A_3$	aspect ratio, $D_h/H$
$B_z$	body force vector in z-direction
$C_p$	specific heat, $\text{W}\cdot\text{s}/\text{kg}\cdot^{\circ}\text{C}$
$D_h$	hydraulic diameter, $2LD/(L + D)$
$g$	acceleration due to gravity, $\text{m}/\text{s}^2$
$\text{Gr}$	Grashof number, $g\beta D_h^3 \delta T/v^2$
$H$	height of chamber, m
$k$	thermal conductivity, $\text{W}/\text{m}\cdot^{\circ}\text{C}$
$L$	width of chamber, m
$p$	pressure, $\text{N}/\text{m}^2$
$\text{Pr}$	Prandtl number, $\mu C_p/k$
$\text{Ra}$	Rayleigh number, $\text{Gr}\cdot\text{Pr}$
$\text{Re}$	inlet Reynolds number, $D_h W_{IN}/v$
$T'$	non-dimensional temperature, $(T_{IN}-T)/(T_{IN}-T_w)$
$T$	dimensional temperature, $^{\circ}\text{C}$
$T_{IN}$	inlet temperature, $^{\circ}\text{C}$
$T_{sw}$	side wall temperature, $^{\circ}\text{C}$ (see Fig. 1)
$T_w$	end wall temperature, $^{\circ}\text{C}$ (see Fig. 1)

<i>t</i>	time, s
<i>u</i>	x-direction velocity, m/s
<i>V<sub>max</sub></i>	magnitude of maximum velocity, <i>v</i> /W <sub>IN</sub>
<i>v</i>	y-direction velocity, m/s
<i>w</i>	z-direction velocity, m/s
W <sub>IN</sub>	inlet vertical velocity, m/s
<i>x</i>	spatial coordinate, m
<i>y</i>	spatial coordinate, m
<i>z</i>	spatial coordinate, m

#### Greek letter symbols

$\beta$	coefficient of expansion, $\beta = -1/p( )$ , 1/K
$\delta$	boundary layer thickness, m
$\delta T$	T <sub>IN</sub> -T <sub>w</sub> , °C
$\mu$	inlet dynamic viscosity, kg/m·s
$\nu$	inlet kinematic viscosity, $\mu/p$ , m <sup>2</sup> /s
$\rho$	inlet fluid density, kg/m <sup>3</sup>

#### INTRODUCTION

Whole-body inhalation exposure chambers with low inlet velocities have traditionally been used to study a variety of chemical atmospheres. Generally, they have a cross-sectional diameter greater than 0.5 m with an inlet velocity set to maintain 10 to 15 chamber volume changes per hour. Initial investigations of whole-body inhalation exposure chambers incorporated flow visualization techniques using smoke and dyes to optimize the chamber geometry so that well-dispersed atmospheres could be obtained (Carpenter and Beethe, 1981; Moss, 1981). Quantitative approaches to determine the mean residence time of a chamber, the rate of dispersion within a chamber of a tracer gas, and the distribution within a chamber using vapors, droplets, and solid particles also have been performed (Hemenway et al., 1982; Beethe, 1979; Moss et al., 1982; Yeh et al., 1986). These investigations, however, have not addressed the effects of mixed convection on the transport phenomenon of heat and mass transfer within these chambers. Information into the diffusion and advection of mass, enthalpy, and momentum within these chambers is of interest to better understand their operation and to provide a basis for improvement, modification, and future development.

This investigation considered a simplified version of the chambers currently used at the Toxic Hazards Research Unit (THRU). Of interest is the development of flow structure due to a temperature gradient between the inlet and wall temperature. Table 6.5-1 shows the scope of this investigation with both two- and three-dimensional numerical formulations and experimental results from a scaled-down laboratory experimental model. The vertical wall temperature, T<sub>w</sub>, was assumed to be constant due to the thin wall construction. A typical range of Reynolds, Grashof, Rayleigh, and Prandtl numbers at the inlet plane, assuming a uniform velocity distribution, also are shown in

Table 6.5-1 where  $\delta T$  is taken to be the difference between the inlet temperature,  $T_{IN}$ , and the vertical wall temperature,  $T_w$ , with the characteristic length chosen to be the hydraulic diameter of the chamber body.

TABLE 6.5-1. PARAMETRIC SPECIFICATIONS FOR NUMERICAL AND EXPERIMENTAL INVESTIGATION

Case #	Approach	$T_{sw}$	$T_w$	$T_{IN}$	$\delta T$
1	2D Numerical	N/A	24 °C	24 °C	0 °C
2	2D Numerical	N/A	24 °C	23 °C	-1 °C
3	2D Numerical	N/A	24 °C	25 °C	1 °C
4	2D Numerical	N/A	24 °C	27.3 °C	3.3 °C
5	3D Numerical	adiabatic	24 °C	27.3 °C	3.3 °C
6	3D Numerical	constant*	24 °C	27.3 °C	3.3 °C
7	3D Experimental	conducting	24 °C	27.3 °C	3.3 °C

Case #	Ra	Gr	Re	$A_1$	$A_2$	$A_3$
1	0	0	235	1.19	0.813	1.31
2	(-) $6.82 \times 10^7$ **	(-) $9.74 \times 10^7$	235	1.19	0.813	1.31
3	$6.82 \times 10^7$	$9.74 \times 10^7$	235	1.19	0.813	1.31
4	$2.25 \times 10^8$	$3.21 \times 10^8$	235	1.19	0.813	1.31
5	$5.84 \times 10^5$	$8.35 \times 10^5$	32.3	1.19	12.2	0.180
6	$5.84 \times 10^5$	$8.35 \times 10^5$	32.3	1.19	12.2	0.180
7	$5.84 \times 10^5$	$8.35 \times 10^5$	32.3	1.19	12.2	0.180

\*  $T_{sw} = (T_{IN} - \delta T)/6$  or  $T' = 1/6$ .  
 $Pr = 0.7$ .

\*\*  $W_{IN} = 4.1 \times 10^{-3}$  m/sec.

\*\* Negative sign signifies "opposed" and positive sign signifies "aided" buoyant force at vertical "end walls."

## ANALYSIS

Both two- and three-dimensional numerical solutions of the incompressible time-dependent laminar Navier-Stokes equations were considered. The time-dependent approach was chosen to evaluate the presence of a steady periodic oscillation. The two- and three-dimensional problems shown in Figure 6.5-1 were considered with the three-dimensional formulation simulating the experimental model. The additional third-dimensional wall is termed "side wall" while the constant temperature two- and three-dimensional vertical wall is termed "end wall."

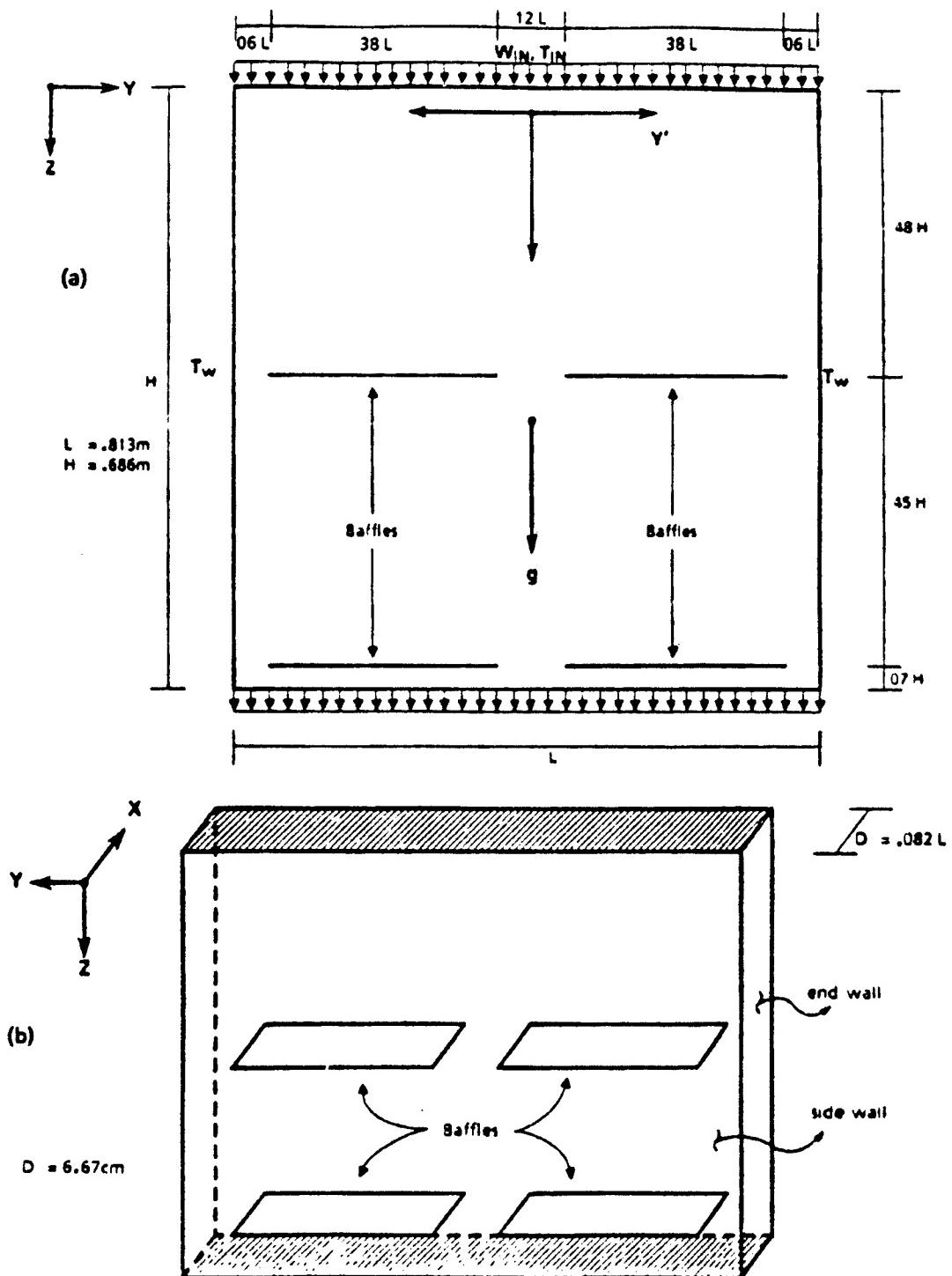


Figure 6.5-1. Chamber Geometry Specifications: (a) Two-Dimensional Geometry and (b) Three-Dimensional Laboratory Model Geometry.

The Boussinesq approximation was used in the vertical direction to account for buoyancy effects. Using this approximation, the vertical body force becomes

$$B_z = \rho g [1 + \beta (T_{IN} - T)]$$

where, for an ideal gas

$$\beta = \frac{1}{T_{IN}}$$

For this investigation, the reference values for all of the gas properties were taken to be those at the inlet plane. The chamber inlet cone was assumed to provide a uniform velocity distribution across the inlet plane with the exhaust manifold providing a velocity distribution resulting from a constant exhaust pressure across the outlet plane. Viscous dissipation and pressure work were assumed to be negligible due to the low buoyancy-induced velocities. With these assumptions, the three-dimensional governing equations become

**Conservation of mass:**

$$\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{\partial w}{\partial z} = 0 \quad (1)$$

**Conservation of momentum:**

$$\rho \left( \frac{\partial u}{\partial t} + u \frac{\partial u}{\partial x} + v \frac{\partial u}{\partial y} + w \frac{\partial u}{\partial z} \right) = - \frac{\partial p}{\partial x} + \mu \left( \frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2} + \frac{\partial^2 u}{\partial z^2} \right) \quad (2)$$

$$\rho \left( \frac{\partial v}{\partial t} + u \frac{\partial v}{\partial x} + v \frac{\partial v}{\partial y} + w \frac{\partial v}{\partial z} \right) = - \frac{\partial p}{\partial y} + \mu \left( \frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2} + \frac{\partial^2 v}{\partial z^2} \right) \quad (3)$$

$$\rho \left( \frac{\partial w}{\partial t} + u \frac{\partial w}{\partial x} + v \frac{\partial w}{\partial y} + w \frac{\partial w}{\partial z} \right) = - \frac{\partial p}{\partial z} + \mu \left( \frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial y^2} + \frac{\partial^2 w}{\partial z^2} \right) + \rho g \beta (T_{IN} - T) \quad (4)$$

**Conservation of energy:**

$$\rho \left( \frac{\partial T}{\partial t} + u \frac{\partial T}{\partial x} + v \frac{\partial T}{\partial y} + w \frac{\partial T}{\partial z} \right) = \frac{k}{C_p} \left( \frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial y^2} + \frac{\partial^2 T}{\partial z^2} \right) \quad (5)$$

#### NUMERICAL SCHEME

The numerical models were formulated using the fully implicit finite-difference of the time-dependent, laminar Navier-Stokes equations using a control-volume approach as developed by Spalding (1980). A combination of the central-difference ( $O(\Delta x)^2$ ) and upwind scheme ( $O(\Delta x)$ ) was used for a hybrid formulation decreasing the numerical errors due to false diffusion for small-cell Peclet numbers ( $Pe$ ) while maintaining numerical stability due to artificial damping for  $Pe > 2$ .

Efforts to verify that numerical solutions were grid and time-step independent involved refining the grid in various locations and reducing the time step. Comparisons of the subsequent numerical results showed a less than 5% variation from the chosen grid size and time step for the reported numerical results.

#### **EXPERIMENTAL APPROACH**

An experimental chamber model was fabricated using 1.91-cm thick plexiglas with a body dimension of 0.813 m  $\times$  6.67 cm and 0.686 m high, as shown in Figure 6.5-2. The vertical end walls were fabricated using a thin aluminum plate combined with a water jacket to maintain a constant wall temperature. Inlet and exhaust air were passed through a series of laminated stainless steel plates with a nominal pore size of 44  $\mu\text{m}$  to maintain a uniform velocity profile. Field temperature measurements were made using iron-constantan-exposed junction thermocouples individually calibrated to within  $\pm 0.1^\circ\text{C}$ . Velocity data were obtained using a TSI IFA-100 hot-wire anemometer with a platinum film sensor model #1211-10 and were limited to the evaluation of the momentum boundary layer thickness along the vertical end wall.

#### **RESULTS AND DISCUSSION**

Experimental data from the experimental model consisted of temperature measurements at various chamber locations at the central plane and hot-wire anemometry data to determine the momentum boundary layer thickness. These experimental data then were compared with results obtained from the three-dimensional numerical solutions, also at the central plane, to simulate this same experimental model. Two-dimensional numerical solutions to simulate the full-size chamber also were compared to the same model experimental data and the corresponding three-dimensional numerical solutions.

Figures 6.5-3 through 6.5-6 show the chamber velocity direction, velocity magnitude, and temperature contours for the two-dimensional cases #1 through 4. Figures 6.5-7 through 6.5-10 show the temperature contours and velocity vector and magnitude plots for the three-dimensional cases #5 and 6. Final comparisons of the non-dimensional temperature,  $T'$ , are shown in Figures 6.5-11 and 6.5-12 for the two-dimensional full-size chamber case #4, the three-dimensional cases #5 and 6, and experimental results case #7. Momentum boundary layer thickness and maximum velocity for the cases investigated are shown in Table 6.5-2.

Three characteristic flow structures were observed for negative, positive, and zero differences between the inlet and vertical wall temperatures. The three-dimensional cases exhibited additional flow structure characteristics inherent to the third-dimensional boundary condition being either fixed temperature or adiabatic.

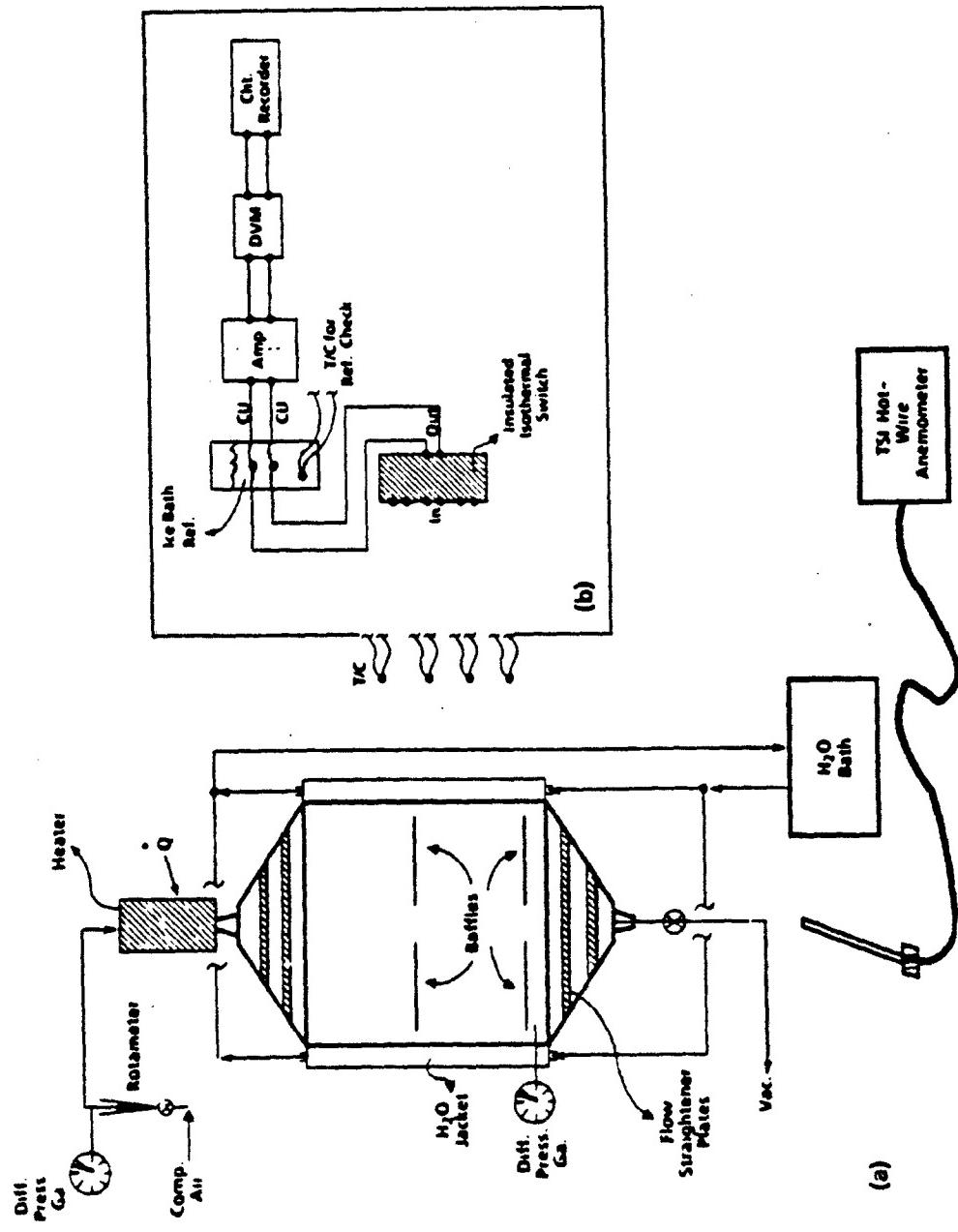
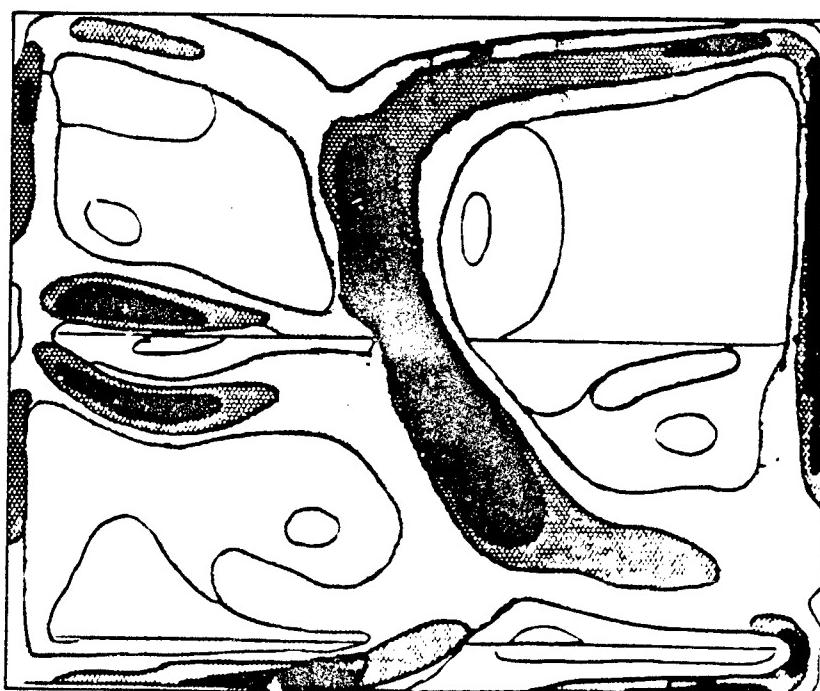
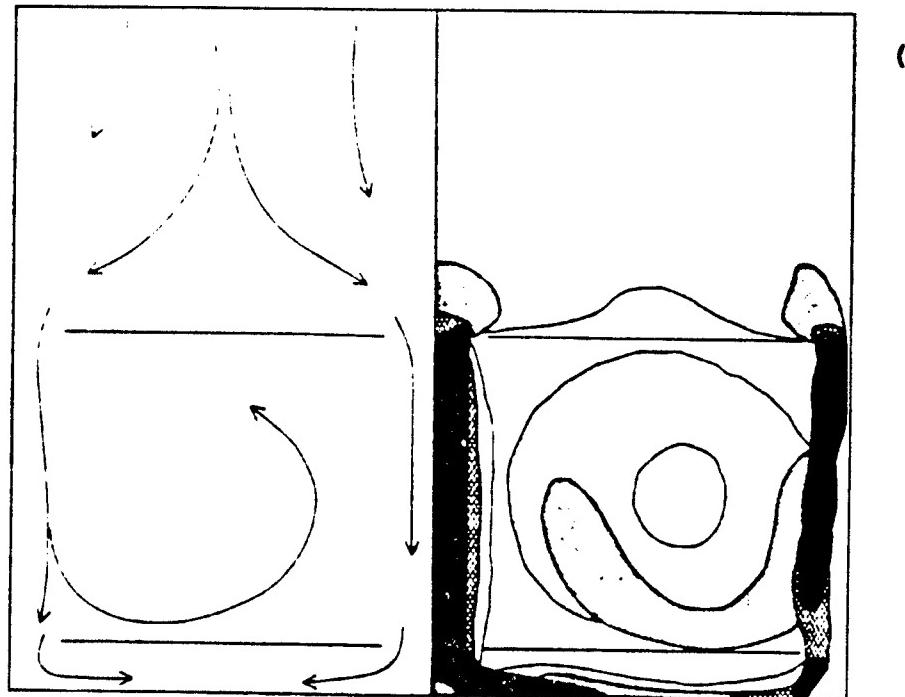
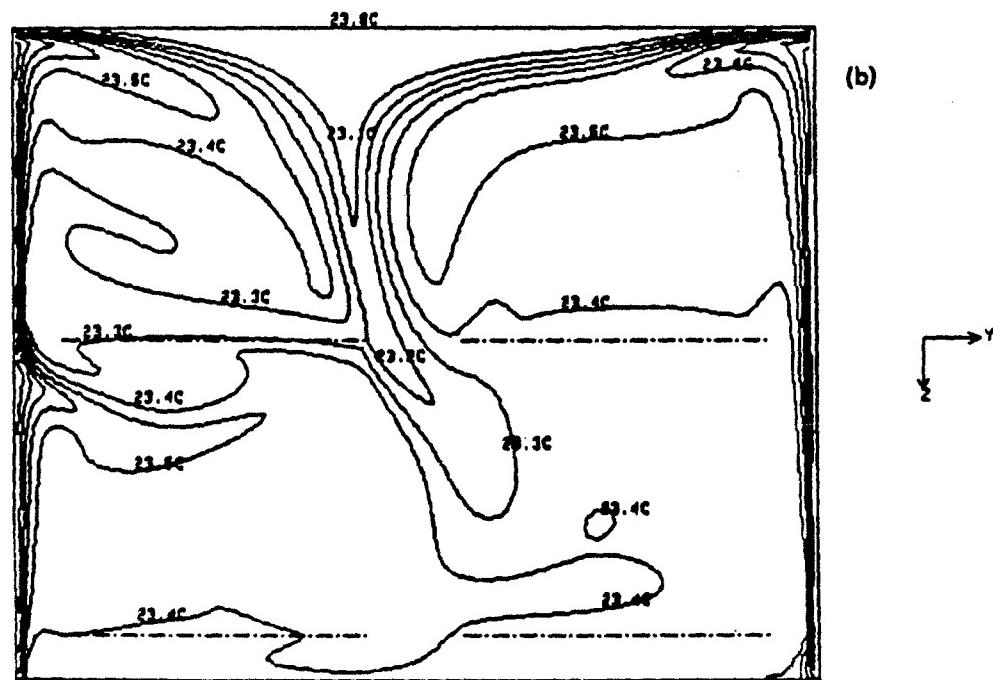
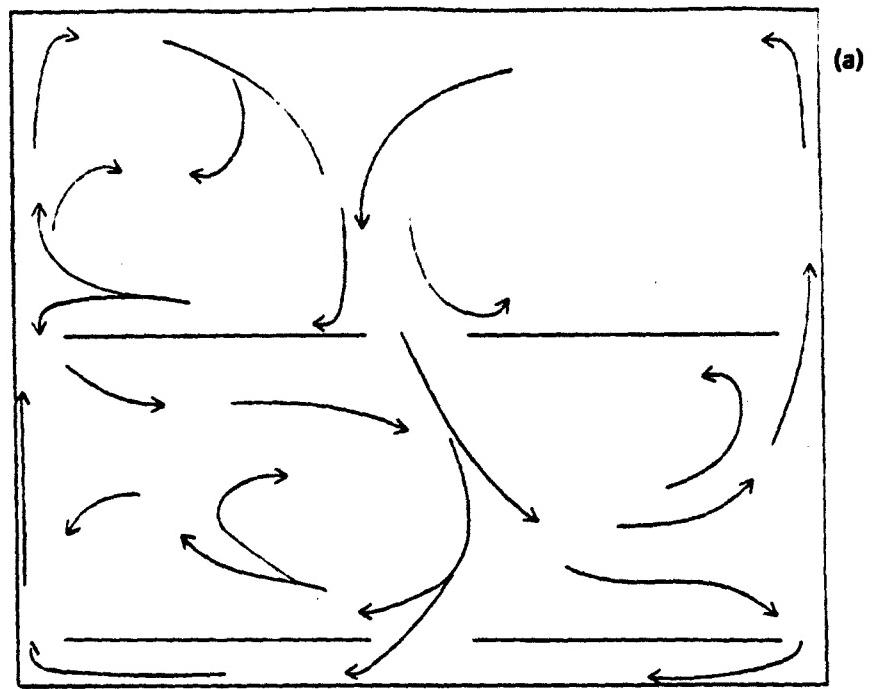


Figure 6.5-2. Experimental Apparatus: (a) Laboratory Experimental Model and (b) Temperature Measurement Setup.



**Figure 6.5-3.** Two-Dimensional Numerical Results for Chamber Velocity Direction and Magnitude Profile: (a) Case #1 and (b) Case #2.



**Figure 6.5-4.** Two-Dimensional Numerical Results for Case #2: (a) Chamber Velocity Direction Profile and (b) Temperature Contour.

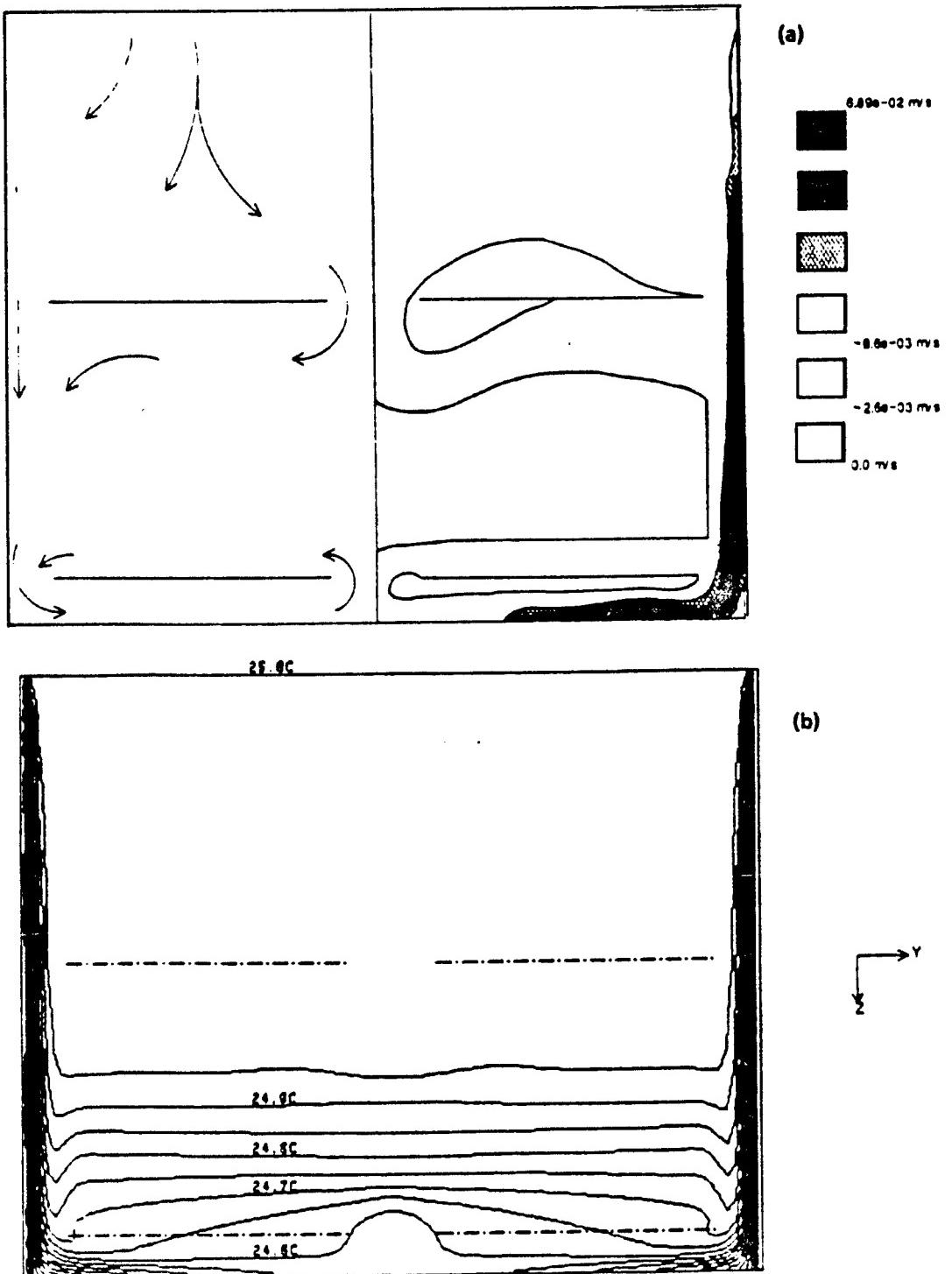
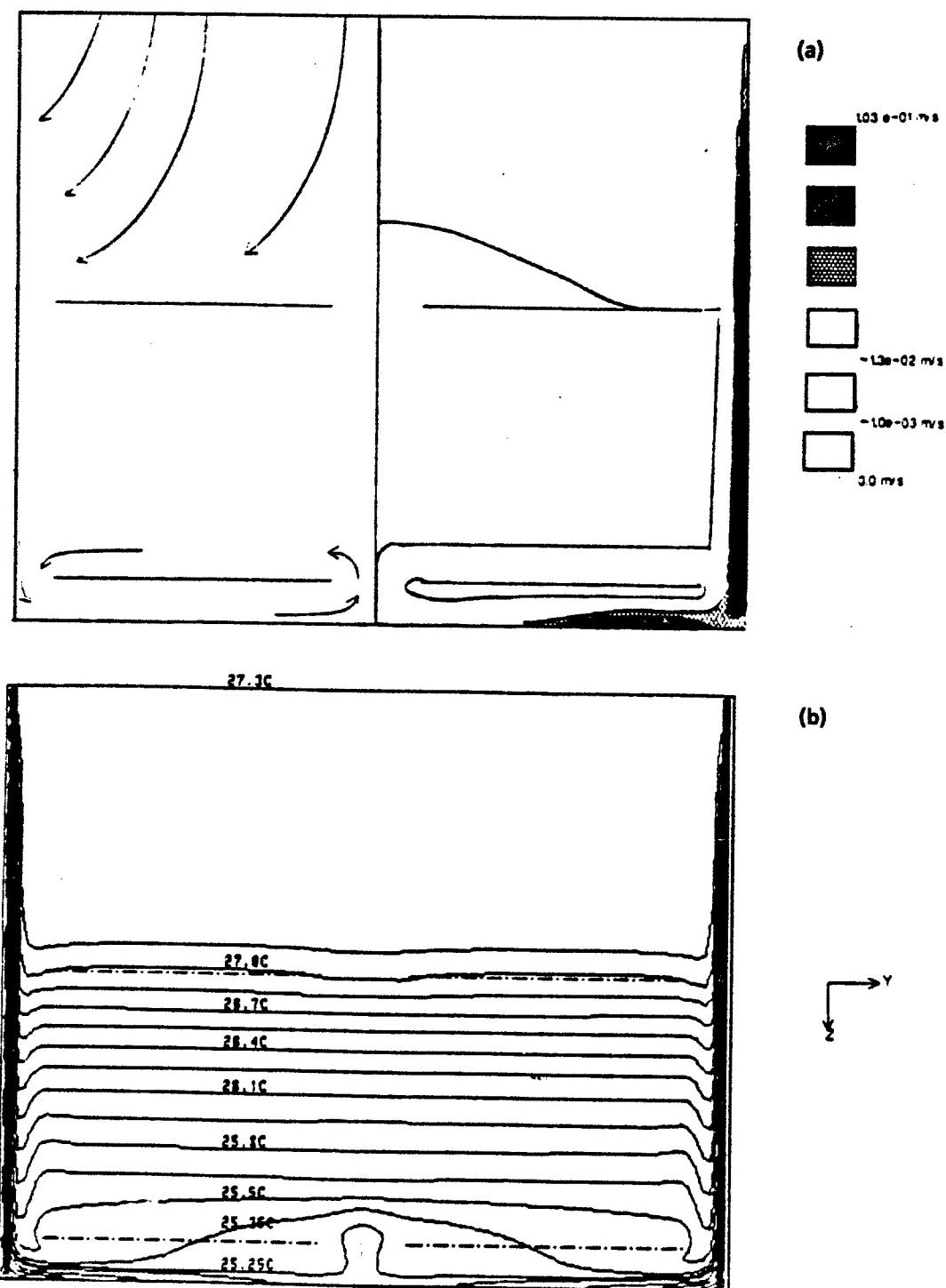


Figure 6.5-5. Two-Dimensional Numerical Results for Case #3: (a) Chamber Velocity Direction and Magnitude Profile and (b) Temperature Contour.



**Figure 6.5-6. Two-Dimensional Numerical Results for Case #4: (a) Chamber Velocity Direction and Magnitude Profile and (b) Temperature Contour.**

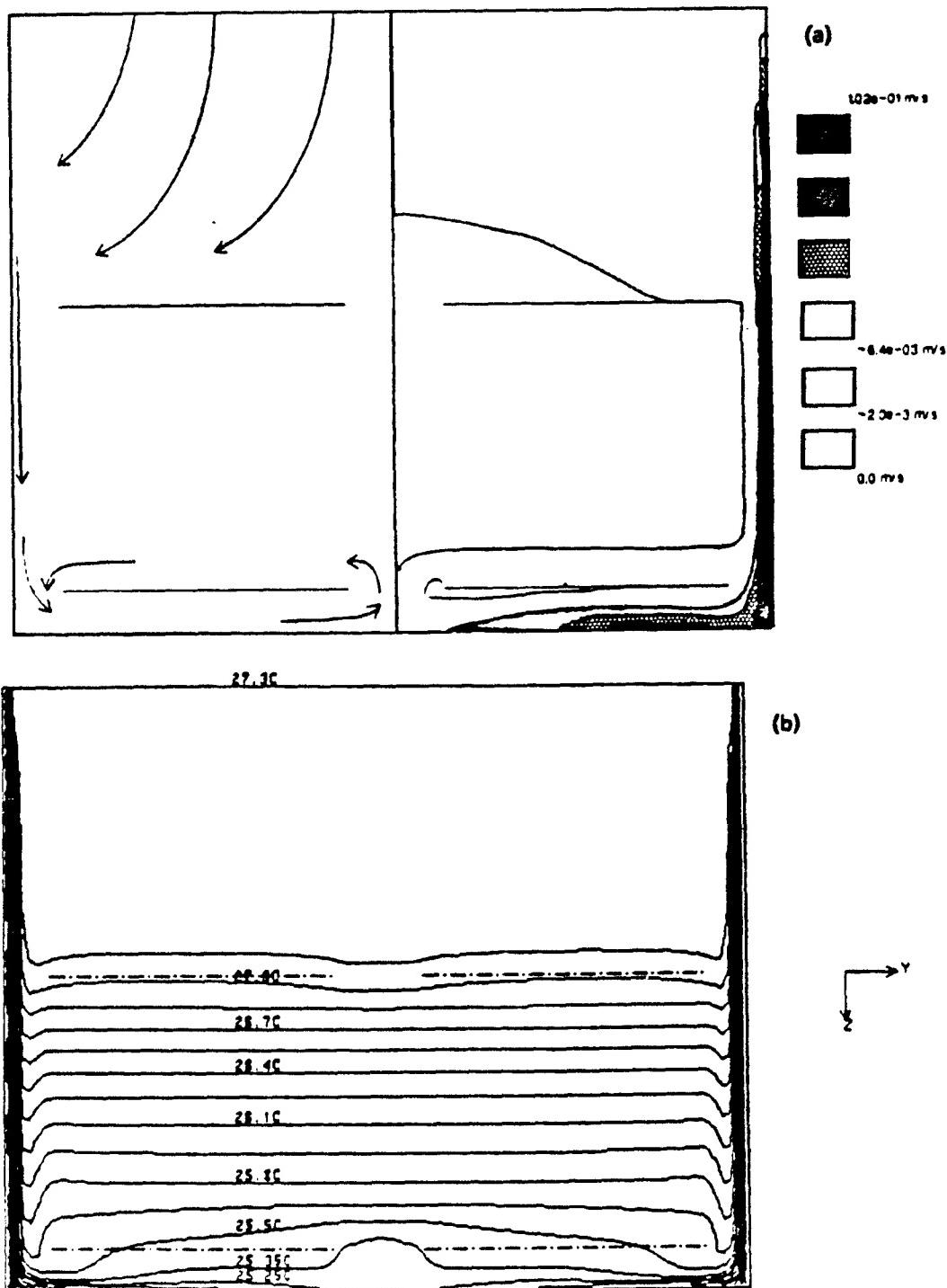


Figure 6.5-7. Three-Dimensional Numerical Results for Case #5 at Center Plane: (a) Chamber Velocity Direction and Magnitude Profile and (b) Temperature Contour.

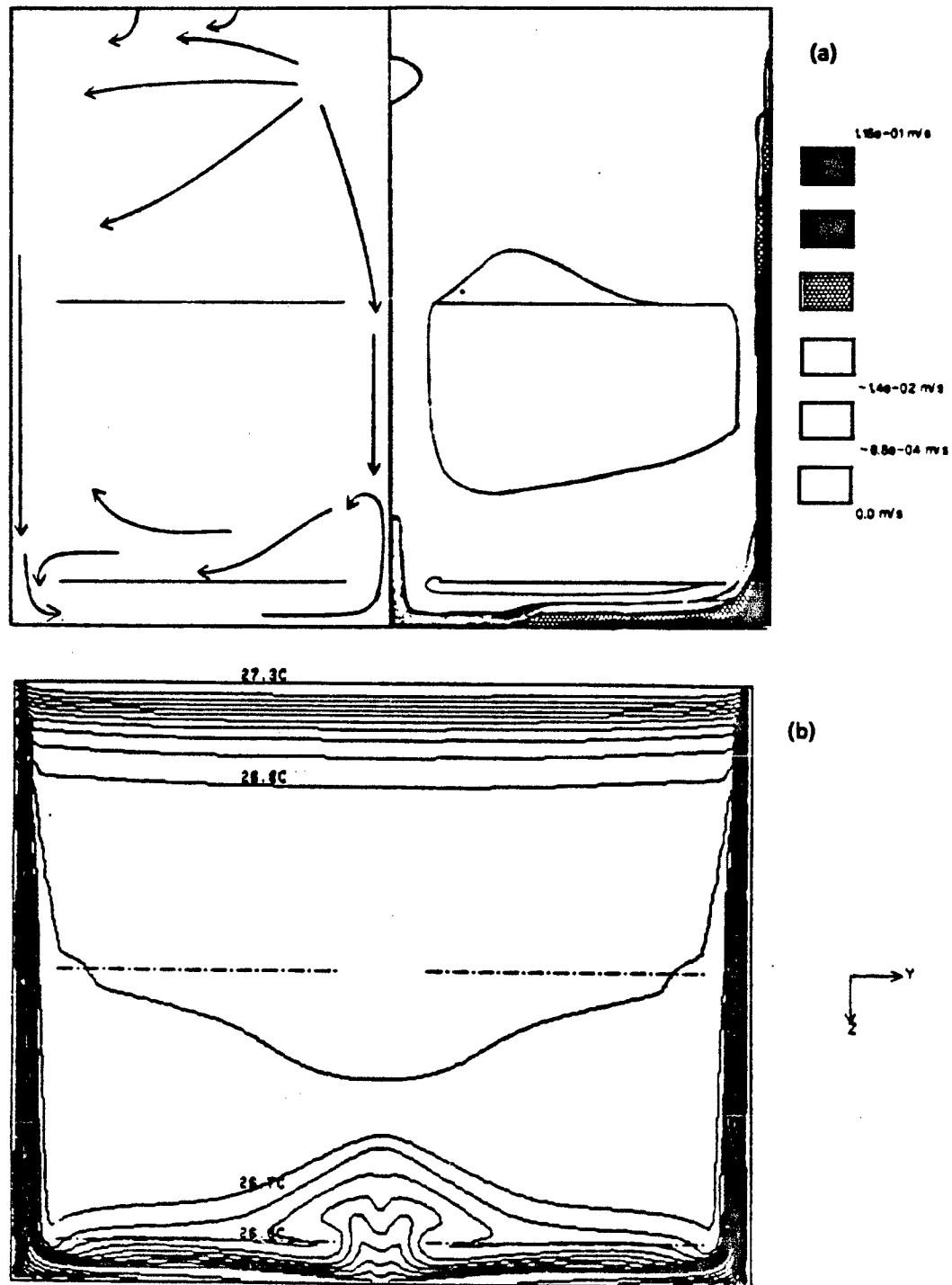
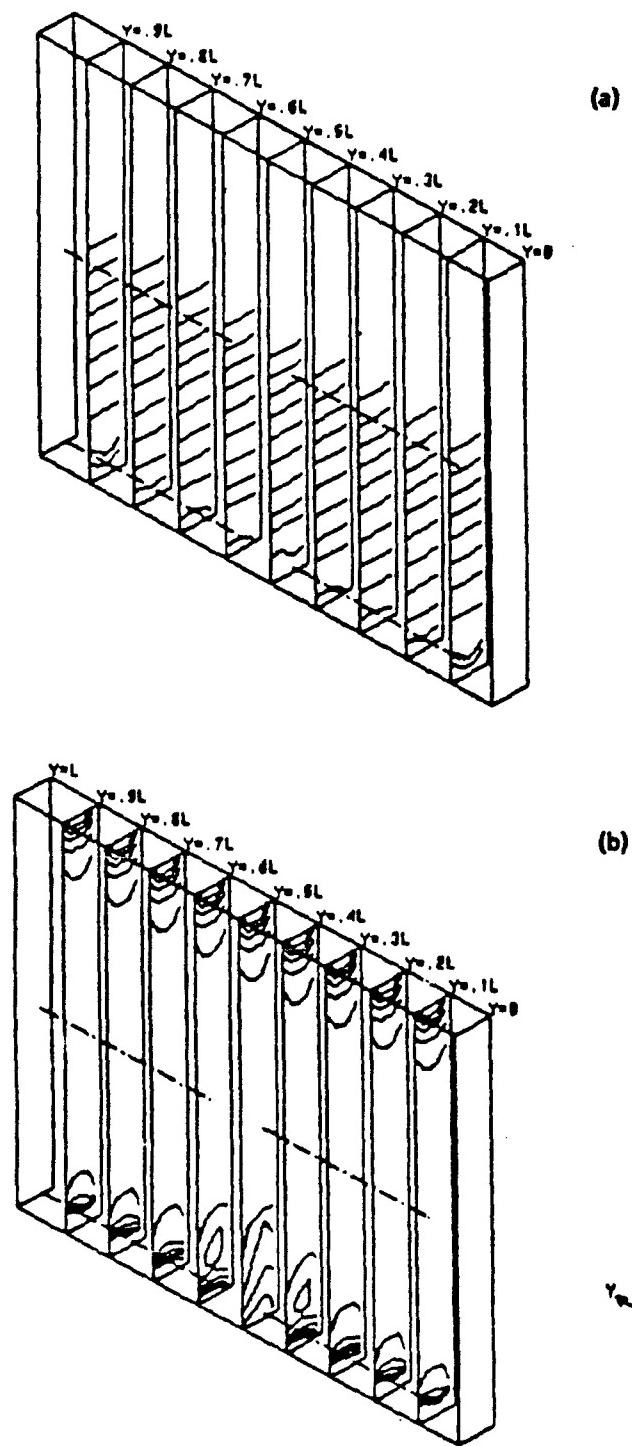
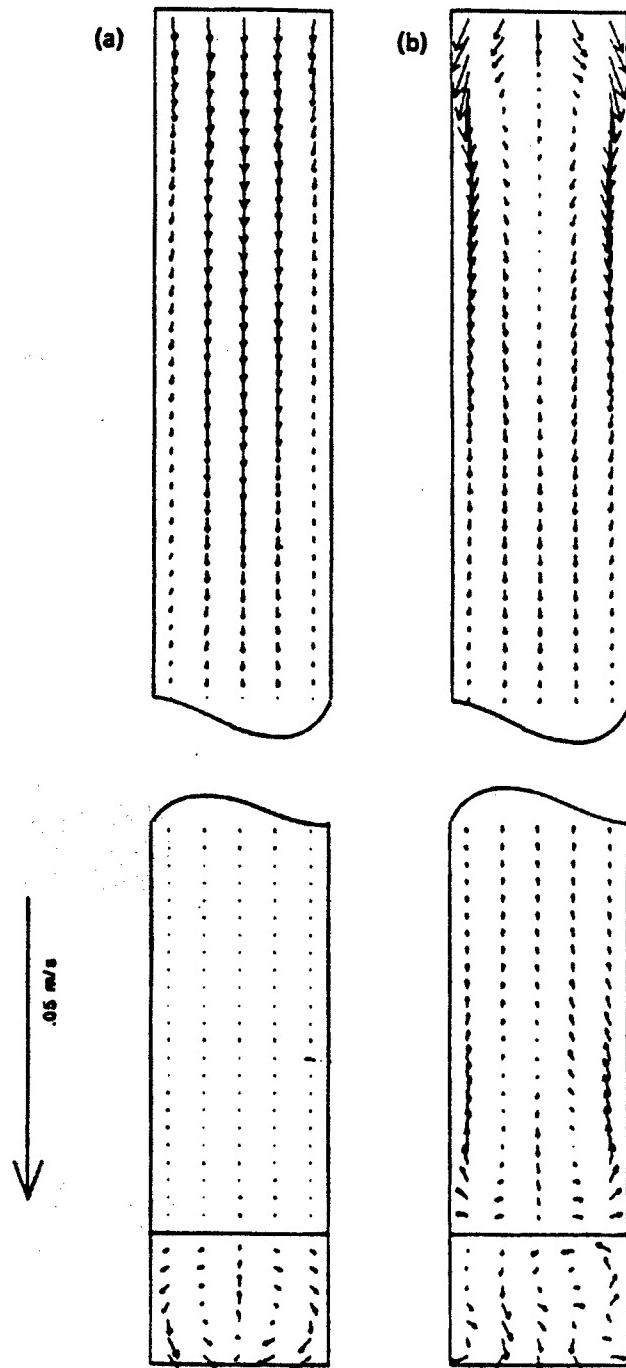


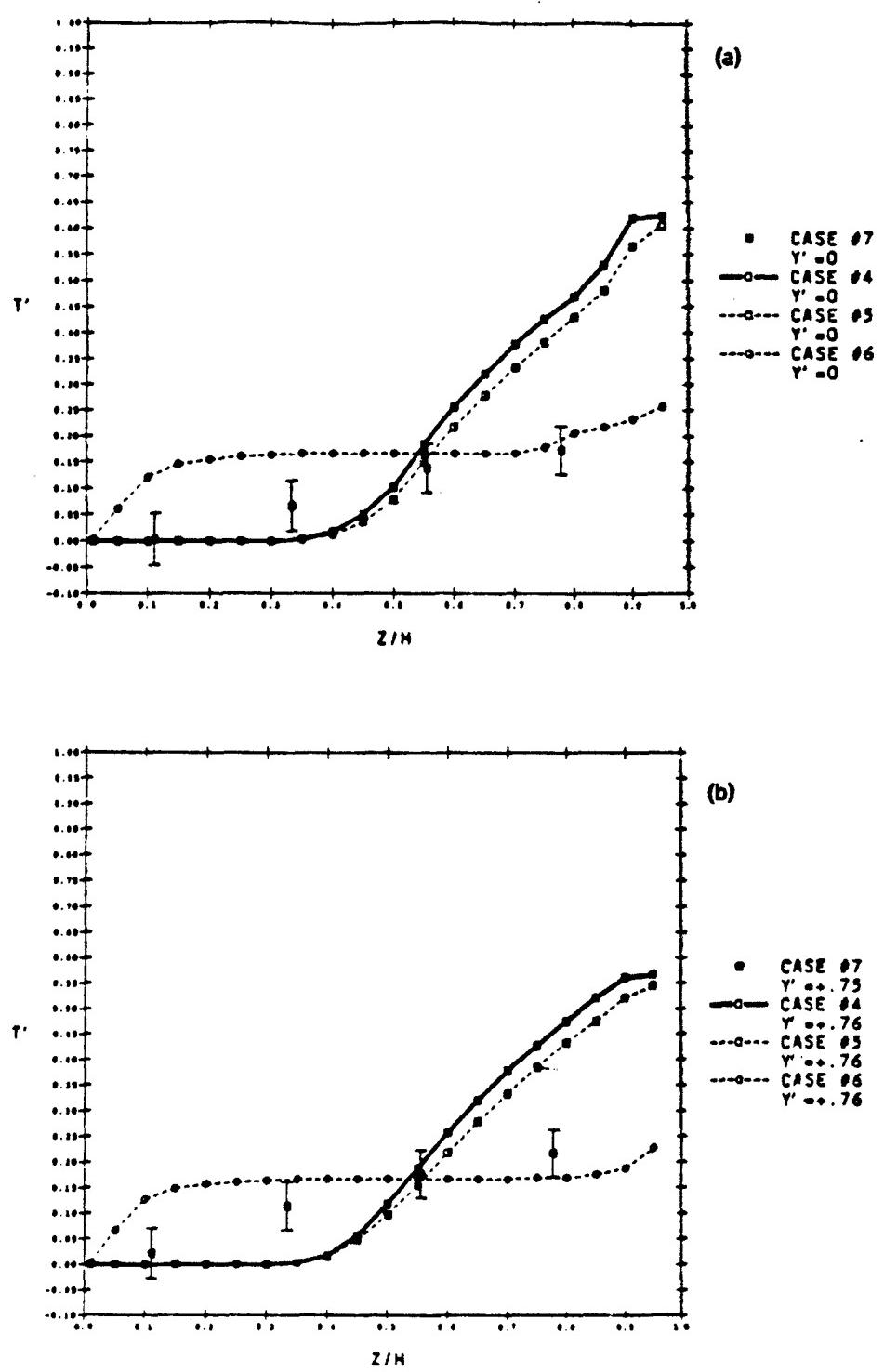
Figure 6.5-8. Three-Dimensional Numerical Results for Case #6 at Center Plane: (a) Chamber Velocity Direction and Magnitude Profile and (b) Temperature Contour.



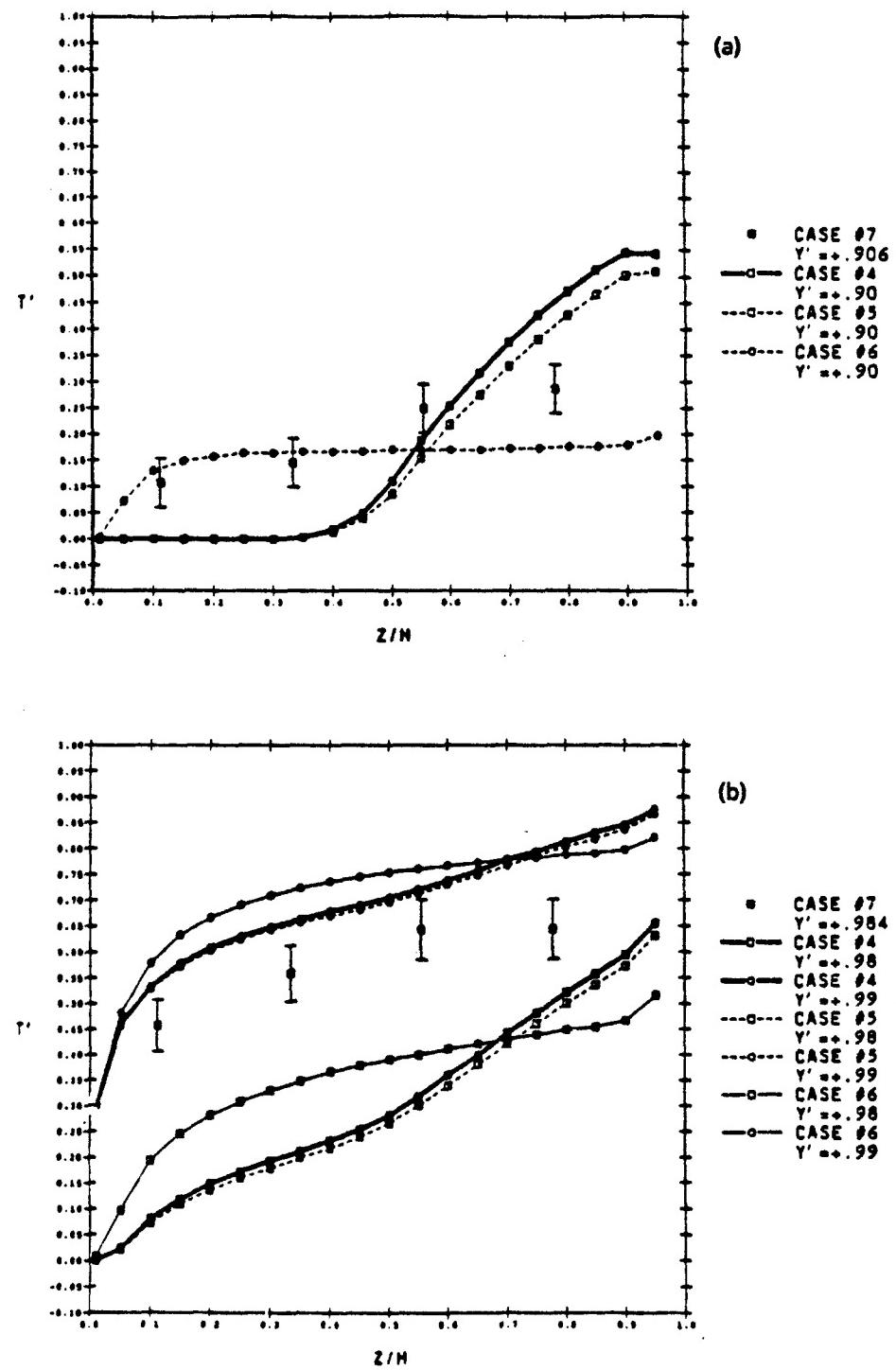
**Figure 6.5-9.** Three-Dimensional Numerical Results, Temperature Contour: (a) Case #5, 25.3 to 27.3 °C at 0.2 °C Intervals and (b) Case #6, 26.3 to 27.3 °C at 0.1 °C Intervals.



**Figure 6.5-10. Velocity Vector Plot Across Depth of Three-Dimensional Numerical Formulation at Lateral Distance 0.25 L as Viewed from the Origin: (a) Case #5 and (b) Case #6.**



**Figure 6.5-11.** Non-Dimensional Temperature vs. Vertical Height for Two and Three Dimensions and Experimental Data along Y'-Axis at Central Plane.



**Figure 6.5-12. Non-Dimensional Temperature vs. Vertical Height for Two and Three Dimensions and Experimental Data along Y'-Axis at Central Plane.**

TABLE 6.5-2. BOUNDARY LAYER THICKNESS AND MAXIMUM VELOCITY

Case #	$V_{max}$	$\delta^*, \text{cm}$			Characteristic
		Z/H = 0.44	Z/H = 0.67	error, cm	
1	$2.81 \times 10^{-2}$	N/A	N/A	N/A	Steady state
2	$7.03 \times 10^{-2}$	N/A	N/A	N/A	Steady periodic
3	$6.89 \times 10^{-2}$	2.38	2.84	$\pm 0.41$	Steady state
4	$1.03 \times 10^{-1}$	1.98	2.32	$\pm 0.41$	Steady state
5	$1.02 \times 10^{-1}$	1.93	2.31	$\pm 0.41$	Steady state
6	$1.16 \times 10^{-1}$	2.09	2.58	$\pm 0.41$	Steady state
7	not measured	1.91	2.54	$\pm 0.32$	Steady state

\*  $\delta^*$  determined to be the location for a velocity equivalent to the minimum response of the anemometer sensor ( $\approx 2.15 \times 10^{-2} \text{ m/sec}$ ).

### CONCLUSION

Development of the chamber flow structure was found to be sensitive to small variations in the temperature difference between the inlet and wall. For positive temperature differences greater than  $1^\circ\text{C}$ , the entry region above the upper baffles is sufficient to provide enough length for the flow to develop and combine in the boundary layer flow. Upper baffle placement in this instance appeared to have minimal effect upon the development of the resulting flow structure. However, with gradually decreasing temperature differences, the baffle placement had significant impact upon the flow structure, eventually resulting in an asymmetric steady periodic behavior.

These variations in flow structure from steady state to oscillatory behavior are consistent with laminar buoyant flow bifurcations for which Prandtl number, geometry, and Rayleigh number are the parameters to influence the flow structure at a specific bifurcation (Yang, 1988). Transition from a stable, steady-state flow structure to one that is periodic in time, aperiodic, and finally chaotic are examples of specific bifurcations that may be expected with variation in Rayleigh number, Prandtl number, and altered geometry. Such appears to be the case with the development of the flow structure in the chambers investigated here. Variation in Rayleigh number due to the difference between the inlet and wall temperature in conjunction with either the baffle placement or chamber aspect ratios provided the means for these flow structure transitions to occur. This resulted in the observed differences between the two-dimensional formulation to simulate the full-size chamber, the three-dimensional formulations, and laboratory experimental model.

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SECTION 7  
ARMY CHEMICAL DEFENSE TOXICOLOGY

**7.1 THE ACUTE INHALATION TOXICITY OF O,O'-DIETHYLMETHYLPHOSPHONITE SPONTANEOUS HYDROLYSIS PRODUCTS: O-ETHYLMETHYLPHOSPHINATE AND ETHANOL**

E.C. Kimmel, K.L. Yerkes, H.C. Higman, and E.R. Kinkead

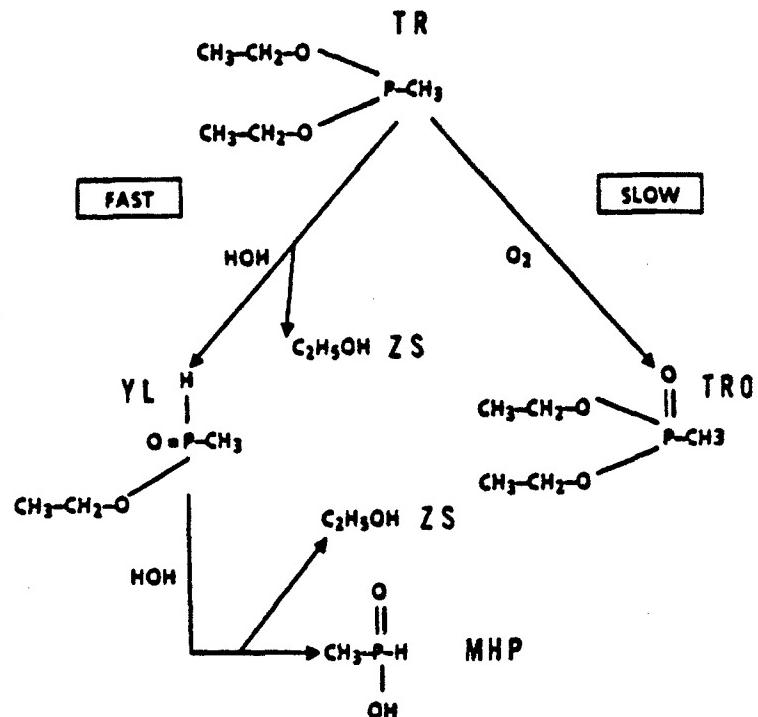
**ABSTRACT**

The virtual acute inhalation toxicity of o,o'-diethylmethylphosphonite (TR) was determined by exposure of two rodent species to its spontaneous hydrolysis products o-ethylmethylphosphinate (YL) and ethanol (ZS). A single 6-h exposure to maximum attainable airborne concentrations of YL and ZS proved to be lethal to mice, whereas rats apparently were not affected.

**INTRODUCTION**

TR is one of several decomposition products of o-ethyl-o'-(diisopropylaminoethyl)methylphosphonite (QL), a component of an organophosphorus chemical munition. Accidental release of QL or associated agents, including TR, may result in untoward effects in exposed personnel. Therefore, determinations have been made of the acute (Vernot et al., 1984) and subchronic (McNamara et al., 1981) inhalation toxicity of QL. The acute toxicity of several compounds associated with QL, including triethylphosphonite (TEP), diisopropylaminoethanol (KB), bis(diisopropylaminoethyl)phosphonite (LT) and TR (Kinkead et al., 1987) also has been determined. The acute inhalation toxicity of TR was not determined due to the necessity to further characterize its chemical instability in air.

Similar to QL (Rohrbaugh et al., 1988), TR undergoes spontaneous hydrolysis in air, forming the primary hydrolysis products YL and ZS in equimolar quantities. TR also undergoes spontaneous oxidation forming the primary oxidation product o,o'-diethylmethylphosphonate (TRO) (Figure 7.1-1). At normal atmospheric relative humidity (RH) the hydrolysis reaction is very rapid, essentially reaching completion within a time frame measured in minutes, whereas the oxidation occurs much more slowly with reaction rates estimated to be on the order of days.



**Figure 7.1-1.** Spontaneous Hydrolysis and Oxidation of TR.

## METHODS AND MATERIALS

### **Test Material**

TR under nitrogen was provided by the U.S. Army Chemical Research, Development and Engineering Center (Aberdeen Proving Ground, MD) for use in this investigation. Chromatographic analyses showed that the material was  $97.12 \pm 1.60\%$  ( $n=9$ ) TR contaminated by  $0.80 \pm 0.83\%$  YL,  $0.34 \pm 0.22\%$  ZS, and  $1.12 \pm 1.21\%$  TRO. The physical properties of TR are shown in Table 7.1-1.

TABLE 7.1-1. PHYSICAL PROPERTIES OF TR

Color and form	Clear to light yellow liquid
Molecular weight	136
Chemical formula	$\text{H}_3\text{CP}(\text{OC}_2\text{H}_5)_2$
Density	0.892 @ 20 °C
Boiling point	119 °C
Flash point	28 °C
Auto ignition point	40 °C
Vapor pressure	11.02 mm Hg @ 21 °C
Other	Strong, noxious odor

### ***Animals***

Male and female Fischer 344 rats (250- to 300-g males, 150- to 200-g females) and CD-1 mice (30- to 40-g both sexes) were obtained commercially for this investigation (Charles River Breeding Laboratories, Raleigh, NC). Both species were fed and watered ad libitum. Food was withheld during exposure. Prior to exposure and during a 14-day postexposure observation period the animals were housed in plastic shoebox cages, kept in temperature- and RH-controlled, laminar air flow facilities maintained on a 12-h light/dark diurnal cycle. Gross pathological examination at necropsy following death or at the 15-day postexposure sacrifice included harvesting the following major organs for weight determination: lungs, liver, kidneys, spleen, brain, spinal cord, stomach, and large and small intestines. Histopathological preparations were made when gross pathological observations warranted.

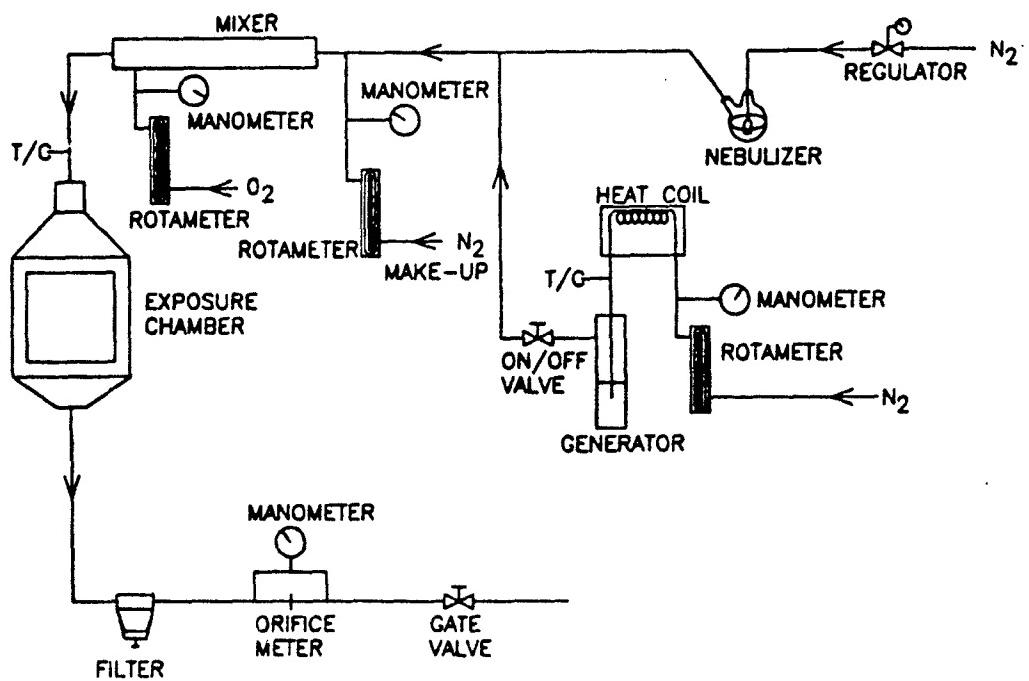
Ten male and female animals each were assigned to control and exposure groups. The investigation required two groups of rats (control and one exposure group) and four groups of mice (control and three exposure groups).

### ***Inhalation Exposures***

In the present investigation, manufactured atmospheres, as opposed to ambient atmospheres, were formed in order to provide control over the hydrolysis and oxidation of airborne TR vapor. Figure 7.1-2 is a schematic representation of the exposure system. Single 6-h inhalation exposures of test animals were conducted using a Wahman 250-L exposure chamber (Wahman Manufacturing, Timonium, MD) operated at 40 L/min air flow. Chamber temperature, RH (Humeter, model 1200D, PhysChem Res., New York, NY), oxygen concentration (EC oxygen monitor, model 5590, Hudson Ventronics, Temecula, CA), and atmospheric pressure were monitored throughout the exposures. The exposure atmospheres were produced by metering TR vapor laden nitrogen into nitrogen carrier gas humidified by compressed nitrogen nebulization of water (Collison, BGI, Waltham, MA). The nebulizer output was 9.5 L N<sub>2</sub>/min laden with 55.4 mg H<sub>2</sub>O/L. Prior to delivery to the exposure chamber pure oxygen was metered into the carrier nitrogen via a gas mixer at flow rates necessary to maintain oxygen concentration at 21%.

### ***Exposure Atmosphere Generation***

An adiabatic, J-tube, sparger evaporator using ultrapure, dry nitrogen carrier gas was used to vaporize TR. Preheated nitrogen (up to 121.1 °C) was delivered (1 to 3 L/min) through a stainless steel sparger (surface area 40 cm<sup>2</sup>) into the J-tube evaporator containing liquid TR. The level of TR was maintained via metered flow ( $\approx$  0.30 mL/min) from a pressurized transfer vessel.



**Figure 7.1-2. Schematic Representation of the Exposure System.**

#### **Atmosphere Characterization and Analysis**

Samples of the exposure atmospheres were collected in a 500-mL gas-washing bottle. Two liters per minute were withdrawn from the exposure chamber through the bottle for 5 min to ensure that an undiluted sample of the chamber atmosphere had been collected. A gas-tight syringe was used to extract 100- $\mu$ L portions of the sample for chromatographic quantification of TR and its hydrolysis and oxidation products. Samples were taken at approximately 20-min intervals. It should be noted that herein mass concentrations of the exposure atmospheres are reported as TR equivalents (mass conversion using molar equivalents based upon measurement of YL); and, on the average, 98% of the phosphorus-bearing material in the exposure atmospheres proved to be YL. Mass spectrometry was used to identify TR-derived components in the chamber atmospheres. In addition, ZS content in the exposure atmospheres was determined chromatographically on samples drawn directly from the chamber.

#### **Statistical Analyses**

Statistical comparisons between the two rat exposure groups were made using a two-sample independent t-test. Comparisons of more than two exposure groups (mice exposures) were made using ANOVA with Bonferroni's multiple t-test (Barcikowski, 1983). The LC<sub>50</sub> of TR in mice was determined using Weil's method (Weil, 1952). Unless specified otherwise all data are reported as the mean plus or minus the standard deviation with the sample number in parentheses.

## RESULTS

### Inhalation Exposures

First-order reaction rates for hydrolysis of TR are shown in Table 7.1-2, at 50% RH the reaction  $T_{1/2}$  for hydrolysis of TR to YL and ZS is 470 msec, thus these two species, with trace amounts of TRO should have dominated the exposure atmospheres. YL hydrolysis products were not found in the exposure atmospheres.

TABLE 7.1-2. FIRST-ORDER REACTION KINETICS FOR HYDROLYSIS OF TR

RH (%)	Rate Constant (min <sup>-1</sup> )	T <sub>1/2</sub> (min)
10	0.00251	277
19	0.00450	154
50	1.45410	0.47

Because the exposure atmospheres were manufactured, general exposure conditions, as well as test material concentration, are reported individually for each exposure.

Exposure of 10 male and female rats each was conducted at a constant 36.8-L/min chamber flow rate at 1.57 mm Hg below ambient pressure. Chamber temperature was  $23.9 \pm 1.8^\circ\text{C}$  ( $n = 20$ ) and the oxygen concentration was 21%. RH at the beginning of the exposure was 20.5%; however as TR concentration began to rise, the RH began to drop steadily to 0.0% after 85 min. The reduction of RH was expected due to water consumption by the TR hydrolysis reaction. Due to the reactivity of TR the exposure concentration was relatively unstable, tending to rise over a period of time far in excess of that predicted by chamber flow/volume relationships (Figure 7.1-3). The mean exposure concentration was  $0.359 \pm 0.255$  mg TR/L ( $n = 15$ ). The contribution of YL to the phosphorus-bearing compounds in the samples ranged from 97.9 to 100%, with traces of TRO present. ZS concentration was not measured during this exposure.

Control exposure of rats was conducted under nearly identical conditions: The chamber temperature was  $20.6 \pm 0.4^\circ\text{C}$  ( $n = 8$ ), the oxygen content was 21%, and the RH ranged from 26.5 to 39.9%.

Exposure parameters for the first exposure of mice to TR were as follows: Chamber flow was 36.9 L/min at 1.40 mm Hg subambient pressure, the temperature was  $24.9 \pm 1.9^\circ\text{C}$  ( $n = 21$ ), and the oxygen content was 21%. Chamber RH ranged from 0.0 to 20.3%. The concentration of TR in the exposure atmosphere was not stable (Figure 7.1-4). The mean exposure concentration was  $0.364 \pm 0.128$  mg TR/L ( $n = 17$ ), with the YL contribution to the phosphorus content ranging from 98.3 to 99.3%, the remainder being TRO. Atmospheric ZS content was  $92.6 \pm 20.39$   $\mu\text{mol}/\text{L}$  ( $n = 16$ ) compared to  $2.8 \pm 0.87$  ( $n = 17$ )  $\mu\text{mol YL}/\text{L}$ , for an average molar ratio of the two species, respectively, of slightly over 33:1 (Figure 7.1-5).

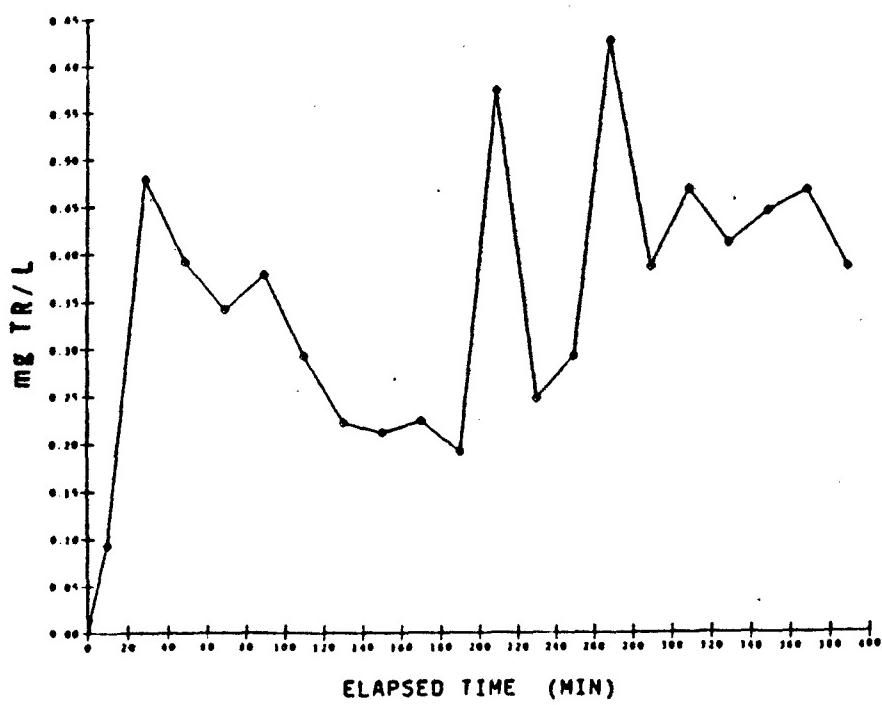


Figure 7.1-3. Concentration vs. Time: 0.359-mg TR/L Exposure of Rats.

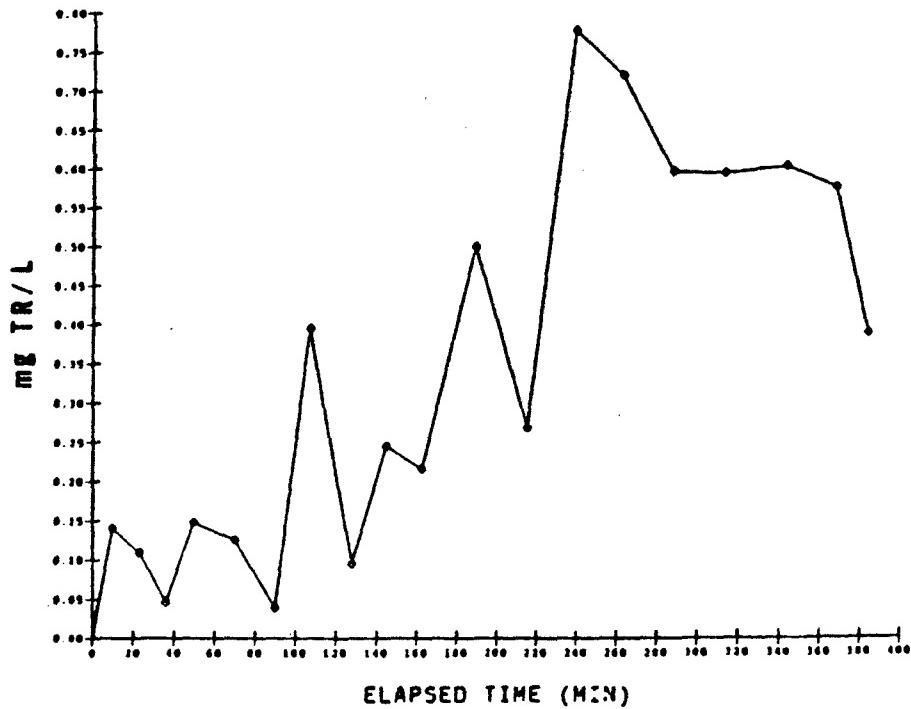


Figure 7.1-4. Concentration vs. Time: 0.364-mg TR/L Exposure of Mice.

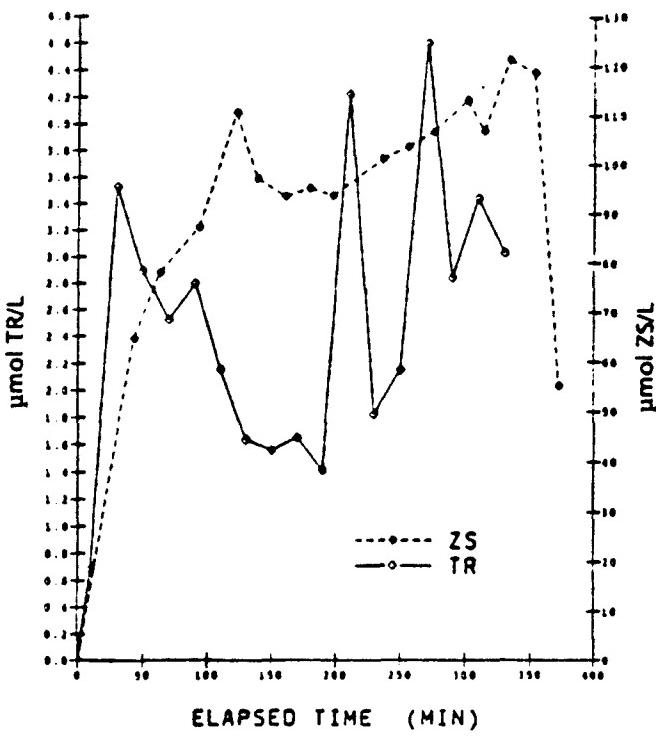


Figure 7.1-5. Molar Concentration of TR and ZS: 0.364-mg TR/L Exposure of Mice.

Exposure conditions for a second exposure of mice to TR were as follows: Chamber flow was 39.2 L/min at 1.59 mm Hg subambient pressure, the chamber temperature was  $16.2 \pm 1.4^\circ\text{C}$  ( $n = 15$ ), and the oxygen content was 21%. RH of the exposure atmosphere started at 22.9% and dropped to 0.0% 150 min into the exposure. The mean concentration was  $0.146 \pm 0.043$  mg TR/L ( $n = 14$ ) (Figure 7.1-6). The samples were found to contain 96.4 to 100% YL, the remainder being TRO. Molar concentration of ZS was  $15.7 \pm 3.41$  ( $n = 14$ )  $\mu\text{mol}/\text{L}$  and was approximately 14 times greater than that of YL, which was  $1.1 \pm 0.31$   $\mu\text{mol}/\text{L}$  ( $n = 14$ ) (Figure 7.1-7).

Exposure conditions for the final exposure of mice to TR were as follows: Chamber flow was 39.2 L/min at 1.59 mm Hg subambient pressure, the chamber temperature was  $16.4 \pm 0.9^\circ\text{C}$  ( $n = 19$ ), and the oxygen content varied from 21 to 22%. RH was relatively low at the beginning of the exposure (6.6%) and decreased to 0.0% in 55 min. Mean exposure concentration was  $0.224 \pm 0.054$  mg TR/L ( $n = 17$ ) (Figure 7.1-8). The exposure atmosphere was 95.1 to 99.6% YL; however, in this case, the remainder of the atmosphere was composed of trace amounts of both TR and TRO. Molar concentrations of YL and ZS were  $1.6 \pm 0.40$  ( $n = 17$ ) and  $29.8 \pm 8.40$  ( $n = 16$ )  $\mu\text{mol}/\text{L}$ , respectively, corresponding to an average molar ratio of 18:1 ZS to YL (Figure 7.1-9).

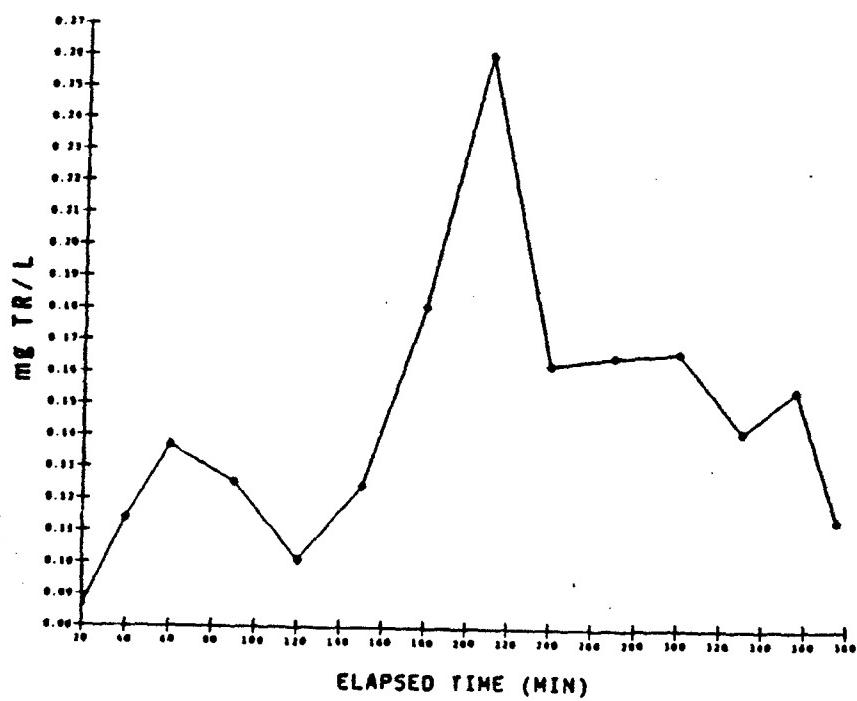


Figure 7.1-6. Concentration vs. Time: 0.146-mg TR/L Exposure of Mice.

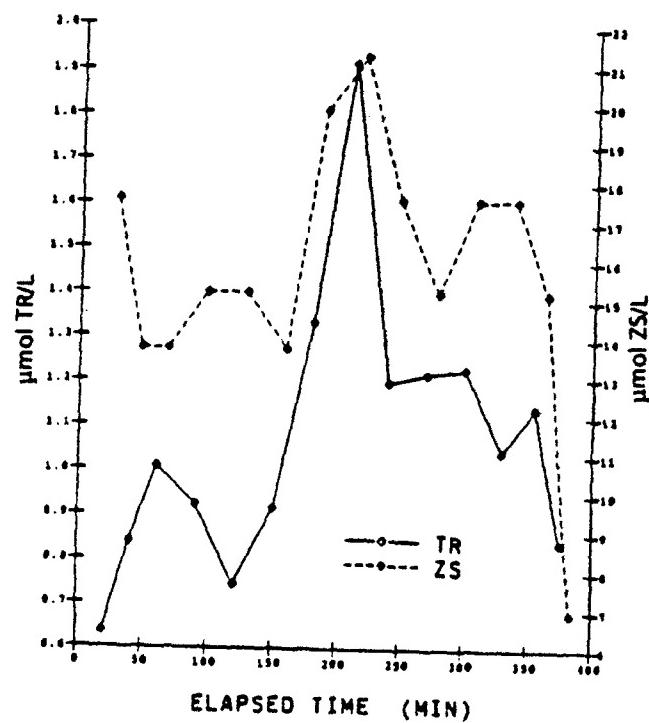


Figure 7.1-7. Molar Concentration of TR and ZS: 0.146-mg TR/L Exposure of Mice.

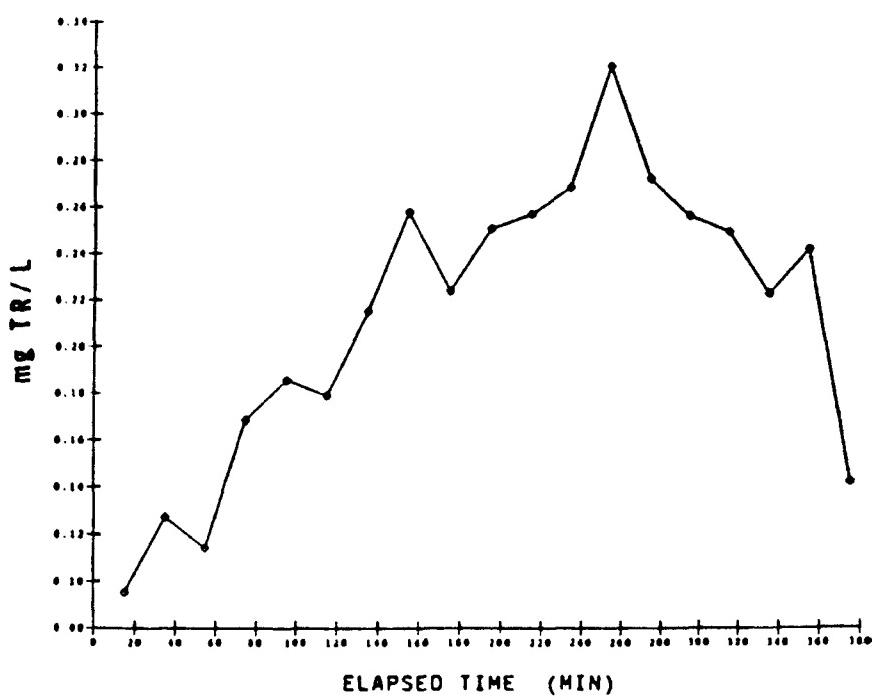


Figure 7.1-8. Concentration vs. Time: 0.224 mg-TR/L Exposure of Mice.

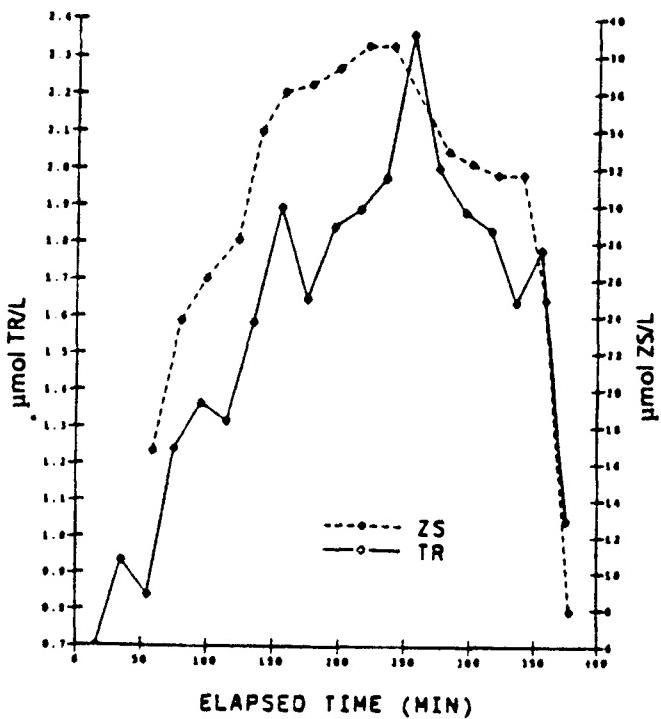


Figure 7.1-9. Molar Concentration of TR and ZS: 0.224-mg TR/L Exposure of Mice.

Chamber atmospheric conditions for the control exposure of mice were similar to those of the TR exposures.

### Toxicity

Exposure of rats to 0.359 mg TR/L was not lethal nor did the exposure affect the general well being of these animals as indicated by postexposure body weight (BW) maintenance. There were no significant differences between terminal BW (15 days postexposure) of exposed vs. controls for either males or females (Table 7.1-3). No gross lesions were observed at necropsy. However, comparison of percent organ weight (OW) to BW ratio did show a significantly larger kidney weight to BW ratio for both exposed males and females (Table 7.1-3).

TABLE 7.1-3. TERMINAL BODY WEIGHTS<sup>a</sup> (BW) 15-DAY POSTEXPOSURE AND PERCENT KIDNEY WEIGHT/BW OF CONTROL AND TR-EXPOSED FISCHER 344 RATS

Parameters	Control Group <sup>b</sup>		0.359-mg/L Group <sup>b</sup>	
	Males	Females	Males	Females
Body Weight	288.9 ± 12.93	183.0 ± 5.22	280.4 ± 15.69	181.9 ± 7.65
Kidney Weight/ Body Weight <sup>c</sup> (%)	0.71 ± 0.042	0.74 ± 0.048	0.77 ± 0.030d	0.78 ± 0.031d

<sup>a</sup> All values are in grams.

<sup>b</sup> N = 10 for males and females.

<sup>c</sup> Kidney weight/body weight.

<sup>d</sup> Significantly different from sex-matched controls at p≤0.05 using a two-sample independent t-test.

Exposure of mice to 0.364 mg TR/L was lethal with death ensuing within 5 days, and males were found to be more susceptible than females (Table 7.1-4). The LC<sub>50</sub> for males was 0.243 mg TR/L (95% confidence interval – 0.184 to 0.321 mg/L); whereas that for females was > 0.364 mg TR/L. The terminal BW of males exposed to both the 0.364 and 0.224 mg TR/L concentrations were significantly lower than the controls; however, no significant differences of terminal BW between groups was observed for females (Table 7.1-5).

TABLE 7.1-4. LETHALITY IN MICE FOLLOWING EXPOSURE TO TR

Exposure Concentration (TR Equivalents-mg/L)	Mortality	
	Males	Females
0.364	7/10	2/10
0.224	0/10	0/10
0.146	0/10	0/10

TABLE 7.1-5. TERMINAL BODY WEIGHT<sup>a</sup> OF CONTROL AND TR-EXPOSED MICE

Sex	Control <sup>b</sup>	0.364 mg/L <sup>b</sup>	0.224 mg/L <sup>b</sup>	0.146 mg/L <sup>b</sup>
Males	40.3 ± 2.39	31.3 ± 5.09 <sup>c</sup>	36.8 ± 2.51 <sup>c</sup>	39.6 ± 1.64
Females	32.0 ± 1.99	28.2 ± 5.03	33.9 ± 2.87	32.0 ± 1.92

<sup>a</sup> All values are in grams.

<sup>b</sup> N = 10 for all groups except male 0.364 mg/L where N = 8.

<sup>c</sup> Significantly different from sex-matched controls at p ≤ 0.05 using the ANOVA with Bonferroni's multiple t-test.

The one significant change in OW/BW ratio observed in male mice exposed to 0.224 mg TR/L was a lower small intestinal weight to BW ratio. Four significant OW/BW differences were observed in females (Table 7.1-6). The liver and gastrointestinal tracts of females exposed to 0.224 mg TR/L were all significantly lighter than those of the female controls. The small intestines of females exposed to 0.146 mg TR/L also were lighter than controls. Interestingly, no effects on OW/BW ratios of females exposed to the highest concentration were observed.

TABLE 7.1-6. ORGAN-TO-BODY WEIGHT RATIOS<sup>a</sup> OF CONTROL AND TR-EXPOSED FEMALE MICE

Organ <sup>b</sup>	Control	0.364 mg/L	0.224 mg/L	0.146 mg/L
Liver	5.19 ± 0.403	4.84 ± 0.558	3.98 ± 0.411 <sup>c</sup>	4.95 ± 0.403
Stomach	1.17 ± 0.146	1.24 ± 0.200	0.90 ± 0.102 <sup>c</sup>	1.01 ± 0.087
Large Intestines	2.15 ± 0.296	1.96 ± 0.263	1.81 ± 0.184 <sup>c</sup>	1.98 ± 0.289
Small Intestines	6.15 ± 0.492	6.85 ± 0.239	4.54 ± 0.493 <sup>c</sup>	4.23 ± 0.292 <sup>c</sup>

<sup>a</sup> All values are percentages.

<sup>b</sup> Organ weight to body weight ratio (OW/BW).

<sup>c</sup> Significantly different from controls, at p ≤ 0.05 using the ANOVA with Bonferroni's multiple t-test.

Although abnormalities of OW/BW ratio for both genders appear to be confined primarily to the 0.224-mg TR/L exposure group, the only remarkable pathological observations made at necropsy were in seven of the 20 mice (both genders) exposed at 0.364 mg TR/L. Of these seven all but one died within six days of exposure. The most common observation (five of seven animals) was mild to moderate hyperemia of one or more gastrointestinal tract organs. Two animals had distended gallbladders accompanied by bile duct occlusion. Three of the animals were dyspneic prior to sacrifice and were found to have congested lungs. In addition, prior to sacrifice four animals exhibited signs of neurotoxicity.

No remarkable lesions were found in central nervous system tissues harvested for histopathological examination. Interpretation of the lesions observed in lung tissues was equivocal. Control and exposed rats were found to have both interstitial and alveolar edema accompanied by

small ectaticies. In large, the interstitial edema consisted of focal transpositions of serum. The alveolar edema observed, though proteinaceous, was nonpurulent and focal. The accompanying vascular congestion lacked concurrent sequelae. In mice, a single observation of interstitial edema with inflammatory cell presence was made. Given the relatively limited incidence of these lesions, their lack of severity and absence of concurrent findings, histopathological confirmation of TR-induced pulmonary insult was marginal.

## DISCUSSION

### Exposure Atmospheres

The original intent was to conduct a limit test exposure of animals to TR equivalents at the regulatory guideline (EPA) concentration of 5 mg/L, then if necessary, to conduct a more thorough determination of the LC<sub>50</sub> of TR. This was not possible due, primarily, to the reactivity of TR in air. Theoretically, exposure to limit test concentrations of TR are possible because the vapor pressure (P<sub>v</sub>) of TR at 21 °C and 760 torr is 11.02 mm Hg. The saturated vapor concentration of TR would be about 79.6 mg/L. Due to the rapid hydrolysis of TR to YL, a more relevant estimate of the saturated vapor concentration would be based on the P<sub>v</sub> of YL, which is 1.03 mm Hg. The saturated vapor concentration of YL would be 5.9 mg/L. The maximum exposure concentration generated in this investigation was only 6.2% of the theoretical maximum YL concentration. The average exposure atmospheres consisted of more than 98% YL with trace amounts of TR and TRO, thus a 1:1 molar ratio of ZS to YL was expected. The average 21:1 molar ratio of ZS to YL observed in the exposure atmospheres would indicate that only about 5% of the expected YL actually was suspended in the exposure atmospheres. Based on ZS concentration and given a 1:1 molar ratio, nearly the maximum possible amount of YL was generated, thus about 95% of the YL was lost in the system. A 95% assumed loss concurs with a measured loss of up to 92% found during system development. Hence, even when employing carefully controlled, saturated vapor generation methods and technologically advanced exposure systems, the experimental exposure of animals to higher concentrations of YL is improbable. System losses of YL, the erratic fluctuation of YL concentration, and prolonged exposure chamber concentration rise times in the present experiment can best be explained by the extraordinary chamber wall loss of YL, due to thermophoretic shunting of chamber flow to the chamber walls (Yerkes, 1990) and the low diffusion coefficient of YL in air (0.099 cm<sup>2</sup>/sec, approximated).

### Toxicity

With regard to acute inhalation toxicity, TR is apparently more potent than QL or other materials associated with its manufacture. Vernot and associates (1984) reported a 6-h inhalation LC<sub>50</sub> of QL-exposed B6C3F<sub>1</sub>-strain male mice to be 3.50 mg/L. Kinkead and colleagues (1987) reported

6-h inhalation LC<sub>50</sub>s for exposure of CD-1 male mice to TEP, LT, and KB to be 6.20, 1.91, and 1.66 mg/L, respectively. By comparison, TR with an LC<sub>50</sub> of 0.24 mg/L in male mice ranges from 26-fold to sevenfold more potent. A comparison of the oral acute toxicities of TR to QL, TEP, LT, and KB reported by the aforementioned studies, does not suggest a comparable differential magnitude of the toxicity (Table 7.1-7); and, in fact, TR is the least toxic by the oral route of administration.

TABLE 7.1-7. ACUTE ORAL TOXICITY<sup>a</sup> OF QL AND ASSOCIATED COMPOUNDS IN MALE MICE

Compound	LD <sub>50</sub> (g/kg)
QL	2.36
TEP	3.72
KB	0.77
LT	1.29
TR	3.74

<sup>a</sup> Data from Vernot et al. (1984) and Kinkead et al. (1987).

The toxicity of ZS is well known; it has been suggested that literature on the biologic and medical effects of ZS is the largest in medical science (Andrews and Snyder, 1986). The direct effects of ZS are numerous; a review of which is beyond the scope of this document. In addition, the potentiation of the toxicity of other xenobiotics by ZS also is well known. A threshold limit value of 1.9 mg ZS/L has been established (ACGIH, 1986). By comparison the average of the highest mouse exposure ZS concentration was  $4.56 \pm 0.848$  mg/L ( $n = 16$ ). Therefore, neither the possibility that ZS was the principal toxicological agent in the present exposures nor the possibility of ZS synergistic potentiation of YL toxicity can be discounted.

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**APPENDIX A**  
**TOXIC HAZARDS RESEARCH UNIT PERSONNEL LIST**

**TOXIC HAZARDS RESEARCH UNIT ORGANIZATIONAL CHART**

**OFFICE OF DIRECTOR**

**Raymond S. Kutzman, Ph.D.**

**Staff:**

**Garrett, Daphne L.**

**DEPUTY LABORATORY**

**DIRECTOR**

**Rory Conolly, Sc.D.**

**ADMINISTRATION**

**Lois A. Doncaster**

**Supervisor**

**Staff:**

**Angell, Mary Ann**

**Roth, Judith M.**

**BIOMETRY**

**Carlyle D. Flemming**

**Senior Statistician**

**Staff:**

**Smith, Kimberly B.**

**QUALITY ASSURANCE**

**Mathias G. Schneider, Jr.**

**Coordinator**

**Staff:**

**Godfrey, Susan M.**

**RESEARCH SUPPORT**

**Patricia M. Fleenor**

**Manager**

**Staff:**

**Brade, Donald W., P.E.**

**Brewer, John A.**

**Courson, David L.**

**Kinney, Lowell E.**

**Smith, Danita H.**

**Sonntag, William B.**

**Stokes, James S.**

**Smutak, Donald**

**SAFETY**

**Larry R. Snelling**

**Health and Safety**

**Representative**

**BIOLOGICAL SIMULATION**

**Allen Vinegar, Ph.D.**

**Manager**

**Staff:**

**Collins, Betty J.**

**Crank, W. David, Ph.D.**

**Gearhart, Jeffery M., Ph.D.**

**Hambel, Jane M.**

**Higman, Howard C.**

**Howe, Stephanie A.**

**King, Greg A.**

**Mahle, Deirdre A.**

**Pollard, Daniel L.**

**Seckel, Constance S.**

**Staats, Dee Ann, Ph.D.**

**TOXICOLOGY**

**Henry G. Wall, D.V.M., Ph.D.**

**Manager**

**Staff:**

**Bailey, Therlo C., Sr.**

**Bennett, Lisa A.**

**Bunger, Susan K.**

**Calderon, Mark A.**

**Deiser, Patricia A.**

**Dille, Susan E.**

**Doarn, Charles**

**Drerup, Joanne M.**

**Frazier, Sharion**

**Frazier, Sherry E.**

**Godfrey, Richard J.**

**Godin, C. Steven, Ph.D.**

**Henson, Bill R.**

**James, Clarence**

**Kimmel, Edgar C., Ph.D.**

**Kinkead, Edwin R.**

**Koehl, Jeffrey L.**

**Leahy, Harold F.**

**Malcomb, Willie J.**

**Miller, Karen S.**

**Neely, Gloria A.**

**Nicholson, Jerry W.**

**Osborne, Jennifer S.**

**Parish, Margaret A.**

**Perkins, Rodney A.**

**Smith, Jessie L.**

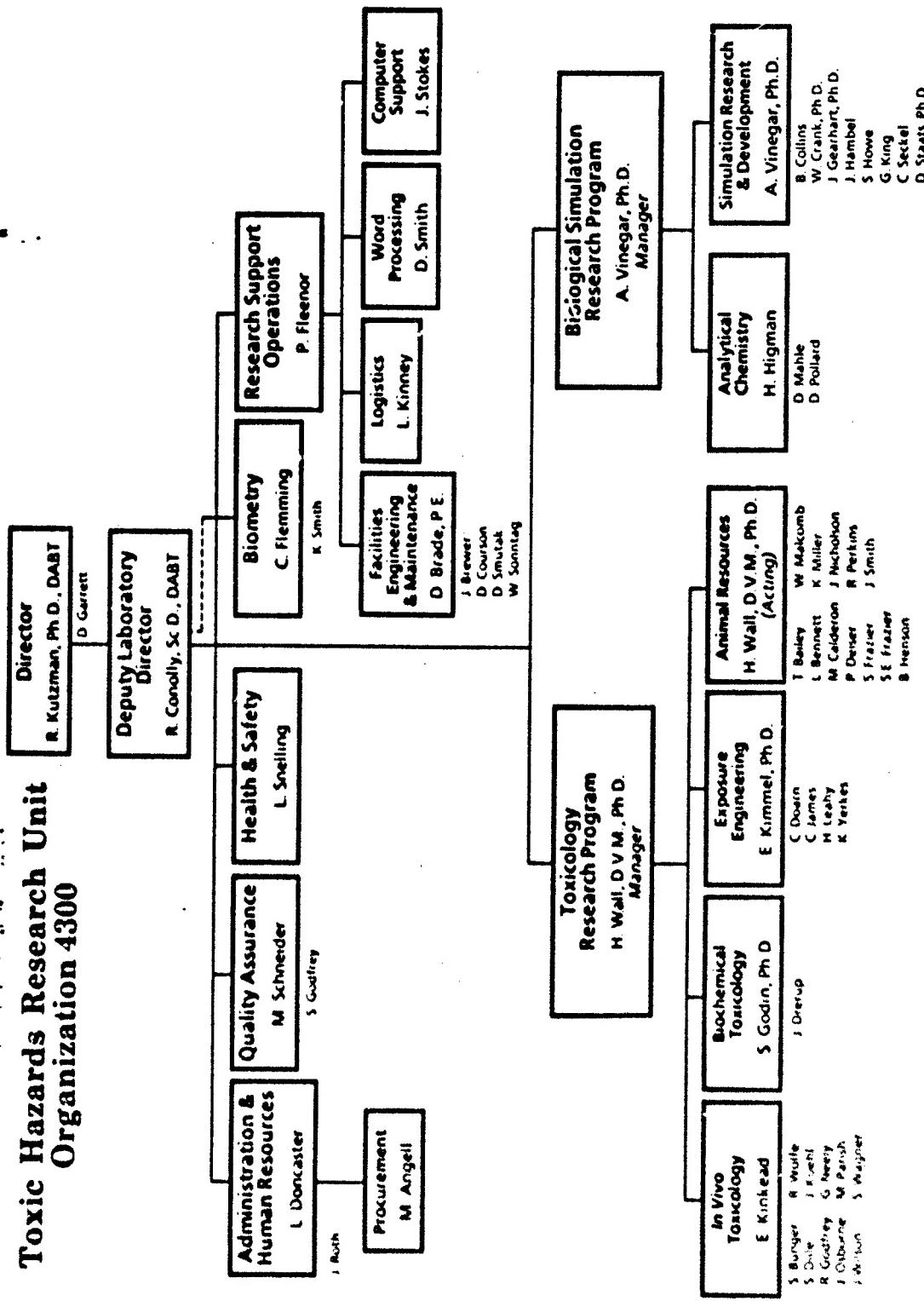
**Wagner, Sharon L.**

**Wilson, Janet L.**

**Wolfe, Robin E.**

**Yerkes, Kirk**

## Toxic Hazards Research Unit Organization 4300



## APPENDIX B

### QUALITY ASSURANCE

This appendix covers the Quality Assurance (QA) activities of the Toxic Hazards Research Unit (THRU) from 1 October 1988 through 30 September 1989.

The QA staff works to maintain records and standard operating procedures (SOPs) in compliance with Good Laboratory Practice (GLP) regulations. Records from THRU studies are microfilmed with backup copies, archived, and are available on an as-needed basis or as required for an audit. SOP manuals and the SOPs themselves are continuously updated as procedures are developed or refined.

An element of the QA program is the NSI-ES QA Manual. The sections of this manual that directly affect the documentation of research at the THRU have been reviewed to assure that they are in concert with local THRU procedures and the interaction of the THRU with the Air Force and Navy.

National meetings have been attended to stay current with GLP regulations and to glean information that will permit the THRU to anticipate future regulations. Such information permits the advanced revision of procedures in a manner that addresses the anticipated change in regulations. The QA of electronic data is one current issue being addressed by GLP regulations. The THRU has formed an Electronic Data Collection Committee that has been charged with the development of procedures for the collection, transfer, and archiving of electronically generated data. The assurance of the integrity of such data is a primary concern of the THRU QA staff.

During this reporting period the THRU was the subject of an NSI-ES systems audit to assure compliance with company standards. Supervisors from each THRU Section were interviewed and personnel were observed during the conduct of selected procedures. The results of this audit were favorable, and several of the procedures developed and implemented at the THRU were acknowledged for their excellence and applicability to other NSI-ES organizations.

The THRU QA Unit completed study report and data audits for the listed Study Requests during the previous year.

<u>STUDY REQUEST</u>	<u>STUDY TITLE</u>
TH 0-10.1	Subchronic Studies on Chlorotrifluoroethylene (CTFE)
TH 0-11.1	Evaluation of the Acute Toxicity of Silahydrocarbon
TH 0-20.6	Ventilation Studies of Perchloroethylene Exposure
TH 0-30.6	Hepatocyte Toxicity of 2,3,4-Trimethylpentane
TH 0-50.4	Paraoxon <i>In Vitro</i> Partition Coefficients
TH 0-51.1	Chloropentafluorobenzene in Expired Breath
TH 0-51.4	Chloropentafluorobenzene (CPFB) Toxicity from Repeated Inhalation Exposure
USN 0-70.1	Cyclotriphosphazene Toxicity from Dermal and 21-Day Repeated Inhalation Exposure
USN 0-80.5	Acute Delayed Neurotoxicity of Four Shipboard Hydraulic Fluids

The QA Unit conducted procedure and data audits during the course of THRU studies listed by Study Request Number.

<u>STUDY REQUEST</u>	<u>AUDIT DATES</u>	<u>TEST PHASE</u>
TH 0-10.1	1-11 October 1988	Data Audit
TH 0-10.6	17, 23 November, 21 December 1988, 25 January, 2, 15 February, 2-16 March, 6, 19 April, 8 June 1989	Physiologically Based Pharmacokinetic (PB-PK) Repeated Exposure
	7, 8, 23 November, 21 December 1988, 25 January 1989	Inhalation Exposure
	7, 21 February 1989	PB-PK Single Exposures
	16, 23 November, 21 December 1988,	Target Organ Exposures

	<b>25 January, 8 February, 10 May, 8 August 1989</b>	
TH 0-10.8	<b>30 January, 1, 2 May 1989</b>	<b>CTFE Oligomer Hepatotoxicity</b>
	<b>18 August 1989</b>	<b>Metabolite Assay Data</b>
TH 0-10.9	<b>31 January, 1 February, 6 March, 10 April, 3 May, 5 June, 10, 20 July, 9 August, 13 September 1989</b>	<b>CTFE Acid Repeated Oral Dose</b>
	<b>1, 15 May 1989</b>	<b>LD<sub>10</sub> Oral Dosing</b>
TH 0-11.1	<b>28 February, 1, 2, 3 March 1989</b>	<b>Skin Irritation</b>
	<b>6, 7, 8, 9, 10 March 1989</b>	<b>Eye Irritation</b>
	<b>27, 29, 31 March, 3, 19, 20, 21 April 1989</b>	<b>Skin Sensitization</b>
	<b>14 June 1989</b>	<b>Data Audit</b>
TH 0-11.4	<b>22 August 1989</b>	<b>GC/MS Data Audit</b>
TH 0-11.6	<b>16-20 October 1989</b>	<b>Skin Irritation</b>
TH 0-20.3	<b>6 February 1989</b>	<b>Partition Coefficients</b>
TH 0-20.6	<b>6 February 1989</b>	<b>Interim Data Audit</b>
TH 0-30.6	<b>3-19 January 1989</b>	<b>Hepatocyte Data Audit</b>
TH 0-30.7	<b>13 February 1989</b>	<b>Data Audit</b>
TH 0-40.2	<b>22 February, 17 August 1989</b>	<b>Interim Data Audit</b>
TH 0-40.4	<b>25 October 1988, 30 January, 8 September 1989</b>	<b>Initiation/Promotion</b>
	<b>28 March, 4 April, 6, 13, 21, 29 June, 15, 22, 29 August, 3 October 1989</b>	<b>CTFE Trimer Acid</b>

TH 0-50.4	9 February 1989 10-14, 21 July 1989	Partition Coefficient Data Audit
TH 0-51.1	17 February 1989	Data Audit
TH 0-51.3	26 July 1989	Interim Data Audit
TH 0-51.4	14 February 1989	Histopathology Specimens
	19 June-2 July 1989	Data Audit
TH 0-51.6	8, 9, 11, 16, 18 May, 7, 14, 15, 20 June, 11, 12, 13 July, 1, 2, 8, 16 August, 6, 25 September 1989	90-Day Repeated Inhalation
TH 0-52.2	13 February 1989	Inhalation Exposure
USN 0-70.1	5 December 1988- 3 January 1989	Data Audit
USN 0-70.3	19, 28 June, 10, 21 July 1989	Acute Delayed Neurotoxicity
USN 0-80.4	8-22 September 1989	Data Audit
USN 0-80.5	3-10 November 1988	Data Audit
USN 0-80.6	9, 13 February, 22 August 1989	Interim Data Audit
USN 0-80.7	26, 27 June, 12 July, 25 August 1989	Dermal Absorption

The QA Unit conducted audits related to THRU organization-wide procedures during the last year.

<u>DATE</u>	<u>PROCEDURE</u>
April, May 1989	Laboratory Notebooks
August 1989	Monthly R&D Report
October 1989	Equipment Logs

QA personnel participated in the review and editing process for manuscripts to be submitted for publication in the peer-reviewed literature, as well as for reports submitted to the Air Force under contract requirements.

<u>STUDY REQUEST</u>	<u>REPORT TITLE</u>
TH 0-10.1	Subchronic Studies of CTFE
TH 0-10.3	Cell Culture Studies on Air Force Chemicals and Materials
TH 0-11.1	Evaluation of the Acute Toxicity of Silahydrocarbon
TH 0-51.1	Development and Application of a Physiologically Based Computer Simulation of CPFB Pharmacokinetics: Quantitation in Expired Breath for Exposure Assessment
TH 0-51.4	Evaluation of the Potential of Inhaled CPFB to Induce Toxicity in F-344 Rats and B6C3F <sub>1</sub> Mice and Sister Chromatid Exchanges and Micronuclei Formation in B6C3F <sub>1</sub> Mice
USN 0-70.1	Determination of the Toxicity of Cyclotriphosphazene Hydraulic Fluid by 21-Day Repeated Inhalation and Dermal Exposure
USN 0-80.5	Evaluation of the Acute Toxicity of Four Shipboard Hydraulic Fluids

## **APPENDIX C**

### **HEALTH AND SAFETY**

Health and safety procedures were implemented to address the changing needs of the Toxic Hazards Research Unit (THRU). These procedures have formed the basis of a Laboratory Health and Safety Manual currently in development.

Hazard Communication (Right to Know) training was presented to meet the requirements of the Occupational Safety and Health Administration Hazard Communication Standard (29 CFR 1910.1200). This training included a presentation by the Safety Representative followed by two videotaped presentations. The purpose of the classes was to train THRU personnel in the safe handling of hazardous materials and to provide manufacturers' safety information for substances used in laboratories. This training will be provided to new personnel as they join the THRU.

The THRU laboratories were subjected to a formal Environmental Compliance Assessment and Management Program (ECAMP) inspection. The ECAMP guidelines were used to determine compliance with environmental regulations and hazardous materials management. THRU personnel used this opportunity to improve labeling of laboratory chemicals and storage cabinets, update each laboratory's chemical inventory, and coordinate waste materials for disposal.

The annual U.S. Air Force grounds safety inspection of the THRU was completed. Safety personnel representing the Armstrong Aerospace Medical Research Laboratory conducted this inspection and will prepare a report of the inspection team's findings. Few deficiencies were noted and corrective actions were initiated to address the deficiencies.

**APPENDIX D**  
**ANIMAL HUSBANDRY TECHNICIAN TRAINING PROGRAM**

The Toxic Hazards Research Unit (THRU) Animal Husbandry technicians are responsible for the care of all animals housed in Buildings 79, 429, 433, 838, and 839 in Area B, Wright-Patterson Air Force Base. Responsibilities include animal care during quarantine and holding, as well as during the pre- and postexposure periods of the in-life phase of scientific studies. Diversified training of the animal husbandry technicians permits additional animal handling and laboratory support capability for the execution of approved research protocols.

The numbers of THRU animal technicians currently certified by the American Association for Laboratory Animal Science (AALAS) are listed below.

Laboratory Animal Technologists	3
Laboratory Animal Technicians	3
Assistant Laboratory Animal Technicians	3

During this year, one technician was certified as a Laboratory Animal Technician and another was certified as an Assistant Laboratory Technician. The outline of the AALAS course was described in detail in a previous THRU annual report (MacEwen and Verno, 1975). All references listed by AALAS that are utilized in preparing for examinations are available through the THRU and Air Force libraries.

To increase the proficiency of new technicians, a special training program has been implemented that will enable new technicians to rapidly acquire proficiency in basic animal technology and animal care routines. This program includes self-study exercises, mandatory reading of animal husbandry standard operating procedures (SOPs), seminars, demonstrations, on-the-job training, and a formal evaluation of animal husbandry knowledge and skills after 60 days of employment. As part of the implementation of this program an adequate supply of up-to-date AALAS animal technology manuals were obtained and an increased library of audiovisual training materials has been developed. A substantial addition to the training material inventory has been the MTM computerized animal technology training program which provides self-paced review and examination on animal technology information relating to all species. This software also is used to prepare computerized examinations on locally developed animal husbandry topics such as information that is included in animal husbandry SOPs.

Ongoing training and education for animal husbandry technicians during the past year included the following.

- Purina Laboratory Animal Care Course, completed by four new technicians.
- In-life computerized animal data acquisition using the Xybion PATH/TOX® system, 12 technicians.
- Nonhuman primate quarantine procedures, three technicians.
- Nonhuman primate pole and collar techniques, 12 technicians.
- AALAS certification training sessions, seven technicians.
- Introduction to AIMS tatoo method, 12 technicians.
- Local training sessions for all technicians on the following subjects.
  - Safety: "Think Snow" film and asbestos monitoring during sandblasting in Building 839.
  - Cage washing, detergents, and temperatures.
  - Personal hygiene.
  - Prevention and management of nonhuman primate bites and scratches.
  - Diseases and restraint of the cat.
  - Effects of selected environmental factors on animal health and research results.
  - Latest developments: Herpes B in monkeys/man.
  - Materials safety training.
  - Correct and legal housing for laboratory animals.
  - The use of physiologically based pharmacokinetic models in toxicological research.
  - Operation and maintenance of the tunnel and automatic cage washers.
  - Laboratory rodent receiving, physical examinations, and acclimation of laboratory rodents to environmental changes.
  - Safety and emergency evacuation procedures.
  - Care and use of temperature recorders.
  - Parasitological examination and parasitic diseases of laboratory animals.
  - "The New Research Environment" Parts I and II videotapes.

A complete list of the programs and training materials used by the animal husbandry technicians is provided below. The numbers in parentheses indicate the percentage of technicians in the current group that have completed these courses.

Purina Animal Care Course - self-study program	(75%)
Biotech Small Animal Series - small animal bleeding, oral dosing, handling, and restraint of laboratory animals	(50%)
Good Laboratory Practices	(67%)
Practical Training List	(33%)
Advanced Practical Training List	(33%)
Toxic Hazards Research Unit Animal Care	(50%)
Laboratory Animal Medicine and Science Autotutorial Series	(50%)
Dome Flight Training	(67%)
Stark/McBride Technologist Correspondence Course	(42%)
Medical Terminology	(25%)
Toxic Hazards Research Unit Parapathologist Video Tapes	(58%)
Laboratory Animal Technologist Video Tapes - preparation for AALAS certification	(17%)
MTM-Animal Technician Training Course	(60%)
Charles River Swine Workshop	(25%)
AIMS Tatoo Method Workshop	(25%)

A cross-training program that was initiated in 1987 was implemented throughout the past year. Technicians were rotated among a variety of animal care and research support assignments on a monthly schedule to increase their capabilities, value, and versatility.

#### **REFERENCE**

MacEwen, J.D. and E.H. Vernot. 1975. *Toxic Hazards Research Unit Annual Technical Report*. AAMRL TR-75-57. Wright-Patterson Air Force Base, OH: Aerospace Medical Research Laboratory.

## APPENDIX E

### SUBMITTED TECHNICAL REPORTS, LETTER REPORTS, AND JOURNAL PUBLICATIONS

#### *Technical Reports*

The Determination of the Acute and Repeated Oral Toxicity of Halocarbon Oil, Series 27-S. E.R. Kinkead, B.T. Culpepper, S.S. Henry, P.S. Szotak, C.D. Flemming, and R.S. Kutzman. 1988. Report No. AAMRL-TR-89-007, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. NMRI-88-16, Bethesda, MD: Naval Medical Research Institute. February.

Conference on Occupational Health Aspects of Advanced Composite Technology in the Aerospace Industry: Volume I and II Proceedings. 1989. Report No. AAMRL-TR-89-008, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. March.

Subchronic Studies of Chlorotrifluoroethylene. E.R. Kinkead, B.T. Culpepper, S.S. Henry, V. Moroughan, E.C. Kimmel, D. Pollard, C.D. Flemming, H.G. Wall, H.F. Leahy, R.S. Kutzman, R.B. Conolly, A. Vinegar, R. Whitmire, and D.R. Mattie. 1989. Report No. AAMRL-TR-89-021, Wright Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. June.

Determination of the Toxicity of Cyclotriphosphazene Hydraulic Fluid by 21-Day Repeated Inhalation and Dermal Exposure. E.R. Kinkead, S.S. Henry, E.C. Kimmel, C.D. Flemming, B.T. Culpepper, C.R. Doarn, H.G. Wall, R.S. Kutzman, J.H. Grabau, and M. Porvaznik. 1989. Report No. AAMRL-TR-89-028, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. NMRI-89-47, Bethesda, MD: Naval Medical Research Institute. September.

1988 Toxic Hazards Research Unit Annual Report. R.S. Kutzman and R.B. Conolly. 1989. Report No. AAMRL-TR-89-022, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. NMRI-89-36, Bethesda, MD: Naval Medical Research Institute. July.

Evaluation of the Acute Toxicity of Silahydrocarbon. E.R. Kinkead, S.K. Bunger, R.E. Wolfe, and C.R. Doarn. 1989. Report No. AAMRL-TR-89-026, Wright-Patterson Air Force Base, OH: Armstrong Aerospace Medical Research Laboratory. September.

#### *Letter Reports*

Evaluation of the Acute Delayed Neurotoxicity of Four Shipboard Hydraulic Fluids. E.R. Kinkead, S.S. Henry, H.F. Leahy, and H.G. Wall. 1989. March.

Evaluation of the Toxic Effects of a 90-Day Exposure to Dimethylphosphonate. H.G. Wall, J. Cramer, C. Hixson, D.R. Mattie, and C.D. Flemming. 1989. June.

Establishing the Cell Culture Capabilities for Future *In Vitro* Studies of Air Force Chemicals and Materials. N.J. DelRaso. 1989. October.

### **Journal Publications**

The determination of the repeated oral toxicity of halocarbon oil, series 27-S. E.R. Kinkead, B.T. Culpepper, S.S. Henry, P.S. Szotak, C.D. Flemming, R.S. Kutzman, R.H. Bruner, J.F. Wyman and D.R. Mattie. *Toxicol. Ind. Health* (In Press).

*In vitro* toxicity of solubilized 2,3,4-trimethylpentane. I. Cytotoxicity and metabolism of TMP using primary hepatocytes. N.J. DelRaso, D.R. Mattie, and C.S. Godin. *In Vitro J.* (In Press).

The study of beryllium oxide genotoxicity in cultured respiratory epithelial cells. V.E. Steele, B.P. Wilkinson, J.T. Arnold, and R.S. Kutzman. 1988. *Inhal. Toxicol.* 1:95-100.

Determination of the toxicity of cyclotriphosphazene hydraulic fluid by 21-day repeated inhalation and dermal exposure. E.R. Kinkead, S.S. Henry, E.C. Kimmel, C.D. Flemming, B.T. Culpepper, C.R. Doarn, H.G. Wall, R.S. Kutzman, J.H. Grabau, and M. Porvaznik. *Am. Ind. Hyg. Assoc. J.* (In Press).

Use of a physiologically based pharmacokinetic model and computer simulation for retrospective assessment of exposure to volatile toxicants. A. Vinegar, D.W. Winsett, M.E. Andersen, and R.B. Conolly. *Inhal. Toxicol.* (In Press).

What's your diagnosis? Red, enlarged, and bulbous nose on a rabbit. S.E. Oille, J. Latendresse, and H.G. Wall. *Lab Anim.* (In Press).

Estimating the risk of liver cancer associated with human exposures to chloroform. R.E. Reitz, R.A. Mendrala, R. Corley, J. Quast, M. Gargas, M.E. Andersen, D.A. Staats, and R.B. Conolly. *Toxicol. Appl. Pharmacol.* (In Press).

Development of a physiologically based pharmacokinetic model for chloroform. R. Corley, R.A. Mendrala, F. Smith, D.A. Staats, M. Gargas, R.B. Conolly, M.E. Andersen, and R.E. Reitz. *Toxicol. Appl. Pharmacol.* (In Press).

### **Journal Publications by NSI-ES Employees (work not conducted at THRU)**

N-Methylation as a toxication route for xenobiotics. II. *In vivo* formation of *N,N*-dimethyl-4,4'-bipyridyl ion (paraquat) from 4,4'-bipyridyl in the guinea pig. C.S. Godin and P. Crooks. 1989. *Drug Metab. Dispos.* 17:180-185.

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Names that are underlined represent NSI-ES employees

## APPENDIX F

### PRESENTATIONS AT SCIENTIFIC MEETINGS

A Physiologically Based Pharmacodynamic Model for Inhibition of Acetylcholinesterase (AChE) by Diisopropylfluorophosphate (DFP). 1989. J.M. Gearhart, G.W. Jepson, H.J. Clewell, M.E. Andersen, and R.B. Conolly. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Acute Pulmonary and Hepatic Toxicity of Inhaled Triethylborane (TEB) Spontaneous Oxidation Products. 1989. K.L. Yerkes, H.F. Leahy, H.G. Wall, and E.C. Kimmel. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Augmentation of Elastase-Induced Emphysema by Cigarette Smoke: Effects of Nicotine Content 1989. E.C. Kimmel and D.W. Winsett. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Augmentation of Elastase-Induced Emphysema by Nicotine Infusion. 1989. D.W. Winsett and E.C. Kimmel. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Chronic Occupational Toxicity of JP8. 1989. C.L. Alden, T.K. Newell, D.R. Mattie, and C.D. Flemming. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Determination of the Subchronic Inhalation Toxicity of Chlorotrifluoroethylene. 1989. E.R. Kinkead, B.T. Culpepper, H.G. Wall, and R.S. Kutzman. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Gastrointestinal Absorption of Xenobiotics in a Physiologically Based Pharmacokinetic Model: A Two-Compartment Description. 1989. D.A. Staats and R.B. Conolly. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Inhalation of Chlorotrifluoroethylene (CTFE) Oil by F-344 Rats: Exposure and Tissue Chemical Analysis. 1989. R.L. Carpenter, E.C. Kimmel, H.C. Higman, and D.L. Pollard. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

Physiologically Based Computer Simulation of Inhalation Exposures of Male F-344 Rats to Chlorotrifluoroethylene (CTFE). 1989. A. Vinegar, D.L. Pollard, E.R. Kinkead, and R.B. Conolly. Presented at the 1989 Annual Meeting of the Society of Toxicology, Atlanta, GA, 27 February-3 March.

A Physiologically Based Computer Simulation of an Isolated Ventilated Perfused Lung: Metabolism of Trichloroethylene. 1989. A. Vinegar, K. Auten, and R.S. Conolly. Presented at the 73rd Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, LA, 19-23 March.

Comparative Hepatic Pathology of Chlorotrifluoroethylene Oligomer, Halocarbon 27-5, and Perfluoro-N-Decanoic Acid in Rats. 1989. H.G. Wall, D.R. Mattie, R.E. Whitmire, and E.R. Kinkead. Presented at the 31st Navy Occupational Health and Preventive Medicine Workshop, Virginia Beach, VA, 11-16 March.

Liver Pathology Following Subchronic Inhalation Exposure of Rats to Chlorotrifluoroethylene Oligomer. 1989 H.G. Wall, D.R. Mattie, R.E. Whitmire, and E.R. Kinkead. Presented at the 31st Navy Occupational Health and Preventive Medicine Workshop, Virginia Beach, VA, 11-16 March.

Determination of the Subchronic Toxicity of Cyclotriphosphazene Hydraulic Fluid by 21-Day Repeated Inhalation and Dermal Exposure. 1989 E.R. Kinkead, L. Goodpaster, B.T. Culpepper, H.G. Wall, and M. Porvaznik. Presented at the 1989 Meeting of the American Industrial Hygiene Association, St Louis, MO, 21-26 May.

Subchronic Inhalation Exposure of Rats to Chlorotrifluoroethylene Oligomer: Clinical and Hepatic Effects. 1989 H.G. Wall, D.R. Mattie, R.E. Whitmire, and E.R. Kinkead. Presented at the Society of Toxicologic Pathologists, VIII International Symposium, Cincinnati, OH, 21-25 May.

Dose-Scaling Across Species. 1989 R.B. Conolly and M.E. Andersen. Presented at the Conference on Environmental Trace Substances, Cincinnati, OH, 29 May-1 June.

Comparative *In Vitro* Cytotoxicity and Metabolism of Solubilized 2,3,4-Trimethylpentane Using Primary Rat Liver and Kidney Cells. 1989 N.J. DeRaso, C.S. Godin, and D.R. Mattie. Presented at the 40th Annual Meeting of the Tissue Culture Association, Orlando, FL, 11-14 June.

Rapid Industrial Hygiene Screening by Headspace GC-MS. 1989 W.R. Sayers and D.L. Geiger. Presented at the 20th Ohio Valley Chromatography Symposium, Houston Woods State Park, OH, 14-16 June 1989.

Species Extrapolation in Physiologically Based Modeling. 1989 R.B. Conolly and M.E. Andersen. Presented at the Fifth International Conference on Environmental Mutagens and the 20th Annual Meeting of the Environmental Mutagen Society, Case Western Reserve University, Cleveland, OH, 10-15 July.

The Effect of 2,3,4 Trimethylpentane on the Ultrastructure of Proximal Tubular Cells in Primary Cell Culture. 1989 D.R. Mattie, J.I. Maslanka, N.J. DeRaso, and M.R. Chase. Presented at the Electron Microscopy Society of America Annual Meeting, San Antonio, TX, 7 August.

Exact Solution for Probability of Tumor (PT) Combined with a Biologically Structured Model for Chemical Carcinogenesis. 1989 R.B. Conolly, D.W. Quinn, H.J. Clewell, and M.E. Andersen. Presented at the Society of Risk Analysis, San Francisco, CA, 29 October-1 November.

An Experimental and Numerical Simulation of Mixed Convection in Large Baffled Rectangular Chambers. 1989 K.L. Yerkes and A. Faghri. Presented at the Winter Annual Meeting of the American Society of Mechanical Engineers, San Francisco, CA, 10-15 December.

**APPENDIX G**  
**INVITED PRESENTATIONS**

**Conolly, R.B.** 1988. Physiologically Based Models and Computer Simulation: What, How, Why?  
Presented at the University of Connecticut, Hartford, CT, 13 November.

**Conolly, R.B.** 1989. Physiologically Structured Models and Computer Simulation: Application to  
Organophosphates. Presented at Rohm and Haas Co., Spring House, PA, 15 June.

**Conolly, R.B.** 1989. Physiologically Based Pharmacokinetic Modeling. Presented at Michigan State  
University, East Lansing, MI, 11 July.

**APPENDIX H**

**1989 TOXIC HAZARDS RESEARCH UNIT (THRU) GUEST SPEAKERS**

<b>DATE</b>	<b>TITLE</b>	<b>PRESENTER</b>	<b>THRU HOST</b>
27 Oct 1988	Cisplatin Pharmacokinetics: Development and Applications of a Physiological Model	Dr. Fred F. Farris	Dr. A. Vinegar
06 Mar 1989	Structure and Dynamics of Iron and Water Permeation through Gramicidin Channels: Brownian and Molecular Dynamics Simulation	Dr. Wing Chiu	Dr. A. Vinegar
13 Apr 1989	Electronic Potential on Dendrites of Neuron Models Using Equivalent Cables	Dr. William Crank	Dr. A. Vinegar
20 Sep 1989	The Effects of the Active Ingredients in Agent Orange in the Guinea Pig and Rat	Dr. Michael Holcomb	Dr. A. Vinegar
28 Sep 1989	Identification of Novel Metabolites of the Anti-Cancer Agent S-Fluorouracil	Dr. David J. Sweeny	Dr. A. Vinegar
23 Oct 1989	Conjugated Metabolites of Ritodrine	Dr. Wayne Brashear	Dr. A. Vinegar